METHODS IN BIOTECHNOLOGY™

Immobilization of Enzymes and Cells

Second Edition

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Cover illustration: Porous solid supports used in "double immobilization" (Fig. 3, Chapter 6; *see* full caption on p. 68 and discussion on p. 67).

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Preface

Enzymes and whole cells are able to catalyze the most complex chemical processes under the most benign experimental and environmental conditions. In this way, enzymes and cells could be excellent catalysts for a much more sustainable chemical industry. However, enzymes and cells also have some limitations for nonbiological applications: fine chemistry, food chemistry, analysis, therapeutics, and so on. Enzymes and cells may be unstable, difficult to handle under nonconventional conditions, poorly selective toward synthetic substrates, and so forth. From this point of view, the transformation—from the laboratory to industry—of chemical processes catalyzed by enzymes and cells may be one of the most complex and exciting goals in biotechnology.

For many industrial applications, enzymes and cells have to be immobilized, via very simple and cost-effective protocols, in order to be re-used over very long periods of time. From this point of view, immobilization, simplicity, and stabilization have to be strongly related concepts. Over the last 30 years, a number of protocols for the immobilization of cells and enzymes have been reported in scientific literature. However, only very few protocols are simple and useful enough to greatly improve the functional properties of enzymes and cells, activity, stability, selectivity, and related properties.

The second edition of *Immobilization of Enzymes and Cells* intends to complement as well as update the first edition. This volume now includes the following aspects of established and new protocols for immobilization:

1. Simple protocols for the immobilization of enzymes and cells that could be very useful for application at industrial scale.
2. Novel protocols for immobilization that can be deployed now or in the near future.
3. Immobilization protocols that can greatly improve the functional properties of enzymes and cells.
4. Different techniques for the characterization of immobilized enzymes and cells as suitable tools for the development of better immobilization techniques.
5. Protocols for the preparation of immobilized derivatives for use in very different nonconventional reaction media.
6. Different protocols for the preparation of immobilized derivatives possibly useful in varied areas of application: therapy, chemical industry, and so on.
7. New chemical reactors that overcome the limitations of a number of immobilized derivatives.

There is still a long and exciting path for developing very simple and efficient protocols for the preparation, characterization, and utilization of immobilized enzymes and cells. *Immobilization of Enzymes and Cells, Second Edition* treats many of the very interesting results already obtained, at the same time giving readers the tools to

develop even more important immobilization protocols. It seems clear that successful development of excellent protocols for immobilization will promote massive implementation of enzyme and cell systems as industrial biocatalysts. Such a development could prove decisive to the emergence of much more skilled and sustainable chemical industry—cost-effective production of very complex and useful molecules under the mildest conditions. The possible advances in fine chemistry, food chemistry, analysis, decontamination processes, and therapeutic applications, for example, only hint at the potential benefits.

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1

Immobilization of Enzymes as the 21st Century Begins

An Already Solved Problem or Still an Exciting Challenge?

Jose M. Guisan

Summary

The main goal of enzyme immobilization is the industrial re-use of enzymes for many reaction cycles. In this way, simplicity and improvement of enzyme properties have to be strongly associated with the design of protocols for enzyme immobilization. In spite of their excellent catalytic properties, enzymes have many other characteristics that are not very suitable for their use in chemical industries: low stability, inhibition by high concentrations of substrates and products, low activity and selectivity toward nonnatural substrates under nonconventional conditions, and so on. The possibility of improving these unsuitable characteristics via the design of simple immobilization protocols is a very exciting goal. There are many protocols for immobilization of enzymes but very few are also very simple and/or very capable of improving enzyme properties. Novel immobilization protocols are still needed in order to achieve a massive implementation of enzymes as catalysts of the most complex chemical processes under the most benign experimental and environmental conditions. A critical review of enzyme immobilization under this point of view is still necessary.

Key Words: Enzymes and sustainable chemical industries; enzyme immobilization protocols; enzyme immobilization techniques; enzyme properties.

1. Advantages and Limitations of Enzymes as Industrial Catalysts

Because of their excellent functional properties (activity, selectivity, and specificity), enzymes are able to catalyze the most complex chemical processes under the most benign experimental and environmental conditions ***(1)***. Enzymes are able to catalyze, under very mild conditions, very fast modifications of a unique functional group (between several similar groups) existing in only one substrate in the presence of other very similar molecules. Therefore, enzymes may be excellent

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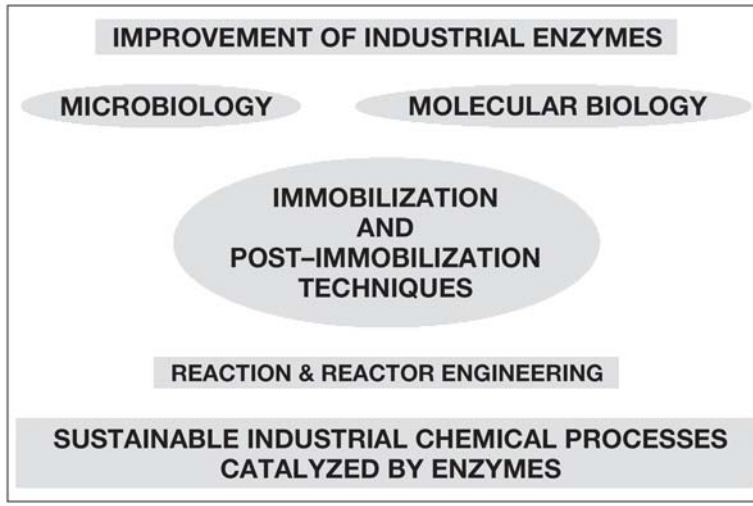

Fig. 1. Biological and chemical tools to improve enzyme properties.

industrial catalysts in a number of areas of chemical industry such as fine chemistry, food chemistry, and analysis.

However, enzymes have been modified during biological evolution in order to optimize their behavior in the framework of complex catalytic chains, inside living things under stress and needing regulation. Obviously, enzymes have not been optimized to work inside industrial reactors. In this way, enzymes, in addition to their excellent catalytic properties, also have some characteristics that are not very suitable for industrial applications: they are soluble catalysts, they are usually very unstable, they may be strongly inhibited by substrates and products, and they only work well on natural substrates and under physiological conditions. In most cases, enzymes have to be greatly improved before their use in industrial processes. The engineering of enzymes, from biological to chemical industries, is one of the most exciting, complex, and interdisciplinary goals of biotechnology. The large-scale implementation of enzymes as industrial catalysts requires a multidisciplinary utilization of very different techniques (*see* **Fig. 1**): (1) the screening of enzymes with suitable properties ***(2)***, (2) the improvement of enzyme properties via techniques of molecular biology ***(3)***, (3) the improvement of enzyme properties via immobilization and postimmobilization techniques ***(4,5)***, and (4) the improvement of enzyme properties via reaction and reactor engineering ***(6,9)***. Such a successful improvement of enzyme properties should be one of the key solutions for the development of a much more sustainable chemical industry that is able to synthesize very complex and useful compounds under very mild and cost-effective conditions.

2. Immobilized Enzymes as Catalysts of Industrial Chemical Processes

For both technical and economic reasons most chemical processes catalyzed by enzymes require the re-use or the continuous use of the biocatalyst for an extended period of time ***(10,11)***. Under this perspective, immobilization of enzymes may be

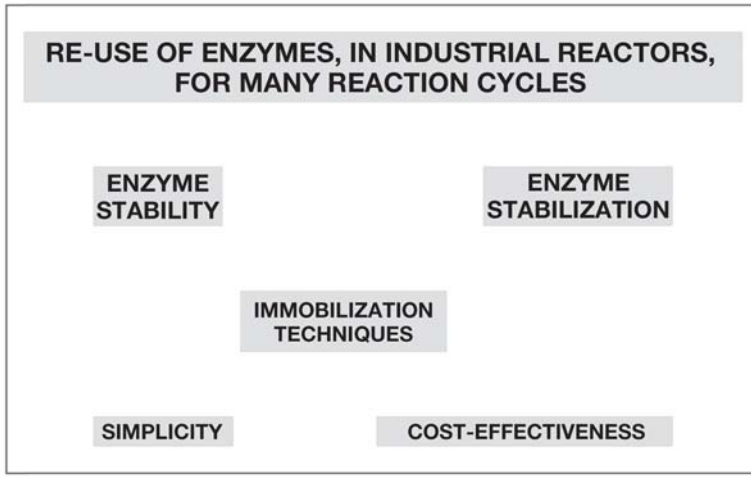

Fig. 2. Factors to be considered in design of a biocatalyst.

defined as any technique that is able to allow the re-use or continuous use of the biocatalysts. From this industrial point of view, simplicity and cost-effectiveness are the key properties of immobilization techniques. On the other hand, a long-term industrial re-use of immobilized enzymes also requires the preparation of very stable derivatives that also have suitable functional properties for a given reaction (e.g., activity and selectivity). At first glance, the practical development of protocols for immobilization of enzymes is intimately related to simplicity, cost-effectiveness and stability, and stabilization of enzymes (*see* **Fig. 2**).

With the above-mentioned parameters in mind, the thousands of protocols for enzyme immobilization that are already reported in scientific literature should be reassessed according to a number of criteria:

1. No need to use toxic or hazardous reagents during and after the immobilization process.
2. The use (in biotechnology companies) of very stable activated pre-existing supports that could have been prepared by other companies with high levels of expertise in synthesis, as well as activation of supports and security protocols.
3. The possibility of associating immobilization of enzymes and improvement of functional properties (activity, stability, and selectivity). In general, we can assume that the preparation of very active and very stable immobilized derivatives is a key requirement for a successful industrial application of such derivatives. Poorly activated and unstable derivatives could be useful for laboratory trials but they are unlikely to be useful for industrial applications. From a practical point of view, very stable enzymes need to be immobilized or enzyme stability needs to be greatly improved as a consequence of their immobilization.
4. The preparation of immobilized derivatives useful in different reactions (e.g., those involving soluble or insoluble substrates, a need for cofactor regeneration, or presence of oxygen as enzyme substrate), in different reaction media (e.g.,

water, organic solvents, supercritical fluids), and in different reactors (e.g., stirred tanks, fluidized beds)
5. The preparation of immobilized derivatives for use in different applications such as fine chemistry, biosensors, and therapeutic applications.

3. The Simplicity of Immobilization of Enzymes

Enzymes can be immobilized in different places for different purposes. In general, it can be assumed that simplicity and the cost-effectiveness of immobilization are not very important when working at the laboratory scale but would be critical for industrial applications.

3.1. Immobilization of Enzymes at the Laboratory Scale

This may be performed for preliminary trials of interesting biotransformations or for more basic structural and functional tests. In these cases, use of toxic or harmful reagents before, during, and after immobilization is not necessary. In addition, unstable activated supports can be used for very rapid immobilization of a low amount of enzyme. Moreover, cost-effectiveness is hardly relevant.

3.2. Immobilization of Enzymes at an Industrial Scale

Many companies (e.g., those in fine chemistry and food chemistry) are able to produce industrial enzymes but are not able—and do not want to be able—to synthesize and activate supports. In these cases, the development of very simple and cost-effective protocols for enzyme immobilization is critical. In addition, the use of very stable and ready-to-use activated supports would also be very convenient.

4. The Improvement of Enzyme Properties Via Immobilization and Postimmobilization Techniques

Functional properties of industrial enzymes can be greatly improved by using suitable protocols for controlled and directed immobilization (*see* **Fig. 3**) ***(12)***. In this way, immobilization of enzymes is a technique necessary for the re-use of enzymes and can also become a very powerful tool for the improvement of enzyme properties. Moreover, enzyme properties could also be improved through physical and chemical modification of immobilized derivatives. Both techniques of enzyme engineering are fairly compatible with additional engineering via preliminary biological techniques (e.g., microbiology, molecular biology) ***(13–15)***.

The following are some protocols for enzyme improvement via immobilization that have already been reported in literature.

4.1. Stabilization of Enzymes by Random Immobilization

Covalent immobilization or strong physical adsorption of enzymes, fully dispersed on the internal surface of porous supports, may promote very interesting stabilizing effects such as ***(16)*** (1) the immobilized enzyme is not able to undergo any intramolecular process (e.g., autolysis, proteolysis, aggregation), and (2) the immobilized enzyme (inside a porous structure) is not able to undergo undesirable interactions with large hydrophobic interfaces (e.g., air/oxygen bubbles, immiscible organic solvents). In this way, under certain experimental conditions, these random immobilization protocols may promote very important stabilizations of

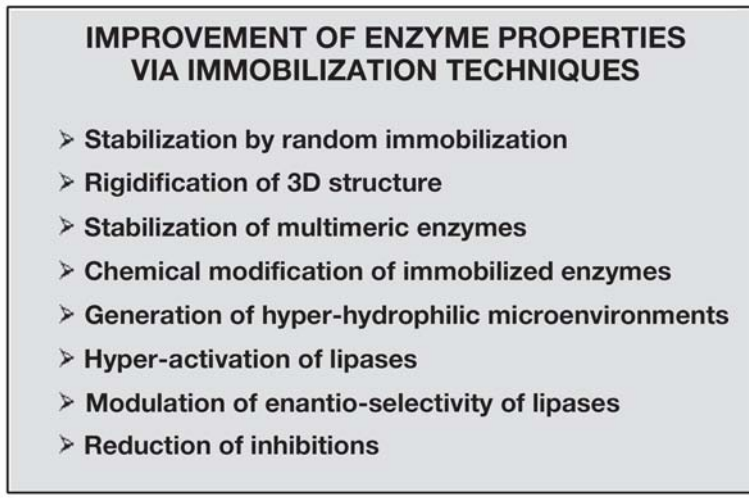

Fig. 3. Possible use of immobilization techniques to improve enzyme properties.

immobilized enzymes with regard to soluble enzymes, as soluble enzymes are able to undergo aggregations and interactions with hydrophobic interfaces.

4.2. Stabilization of Enzymes by Multipoint Covalent Immobilization

Multipoint covalent attachment of enzymes on highly activated pre-existing supports (*see* **Fig. 4**), via very short spacer arms and through a number of residues on the enzyme surface, can promote dramatic stabilization of the three-dimensional (3D) structure of the immobilized enzyme. In this case, the relative distances among all residues involved in multipoint immobilization have to be maintained unaltered during any conformational change induced by a distorting agent (e.g., heat, organic cosolvents, extremes of pH values). In this way, the intensity of conformational changes involved in enzyme inactivation may be strongly reduced and the immobilized enzyme may become strongly stabilized. In fact, a number of enzymes have been dramatically stabilized by multipoint covalent immobilization as compared with one-point immobilized counterparts ***(17–20)***.

4.3. Stabilization of Multimeric Enzymes by Multisubunit Immobilization

Some multimeric enzymes, under certain experimental conditions, may be inactivated via dissociation of the subunits of their quaternary structure. Some protocols for enzyme immobilization may prevent dissociation of subunits and may therefore result in dramatic stabilizing effects ***(21)***.

4.3.1. MultiSubunit Covalent Attachment on Pre-Existing Supports

Both subunits of dimeric enzymes may be easily attached to the support when very highly activated supports are used, resulting in very important stabilizing effects ***(22)***. Of greater difficulty is the stabilization of more complex multimeric enzymes. In this case, several subunits (two or three) can be covalently attached to

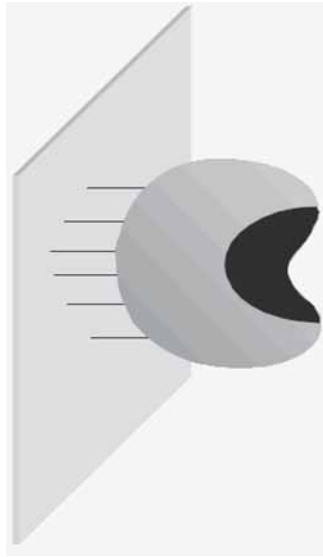

Fig. 4. Multipoint covalent attachment of proteins.

the support, after which other subunits of each enzyme molecule can be cross-linked to the immobilized subunits via bifunctional, or polyfunctional, cross-linking agents (e.g., glutaraldehyde, aldehyde–dextran). In this way, complex multimeric structures can be fully stabilized ***(4,5)*** (*see* **Fig. 5**).

4.3.2. CLEAs or CLECs of Multimeric Enzymes

The aggregation or crystallization of multimeric enzymes with further cross-linking of the aggregated or crystallized beads with bifunctional or polyfunctional reagents should prevent the dissociation of subunits. These protocols for immobilization of enzymes with no support may be very powerful tools for greatly improving the stability of multimeric enzymes ***(23)***.

4.4. Hyperactivation of Lipases by Selective Adsorption on Hydrophobic Surfaces

The adsorption of lipases at very low ionic strength onto hydrophobic supports can promote the immobilization and stabilization of the open structure of lipases (*see* **Fig. 6**) ***(24–26)***. In this way, these immobilized lipases may exhibit a very important hyperactivation—at least towards small and hydrophobic substrates—associated with the immobilization process .

4.5. Physiochemical Modification of Immobilized Enzymes

Immobilized enzymes can be easily modified with chemical reagents ***(27)***. The process of modification can be easily controlled by ending the reaction via fast separation of immobilized enzymes and reagents. Intermolecular chemical modifications are then impossible, and the potential aggregations promoted by the modi-

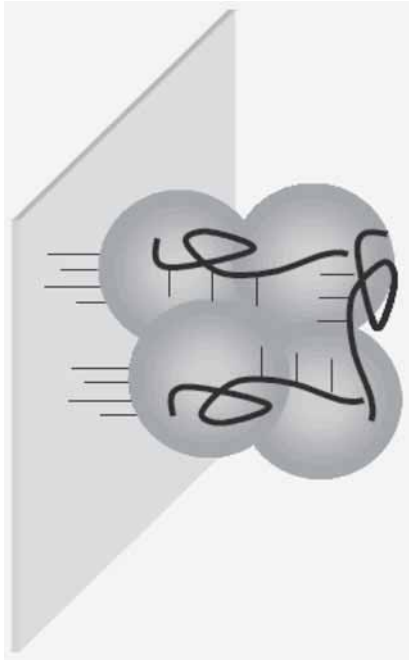

Fig. 5. Stabilization of multimeric enzymes: multisubunit covalent immobilization plus additional cross-linking.

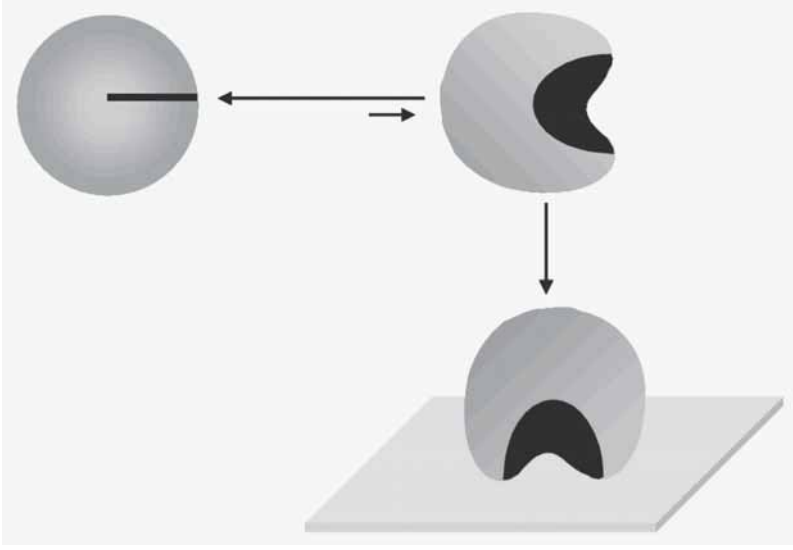

Fig. 6. Hyperactivation of lipases by interfacial activation on hydrophobic supports.

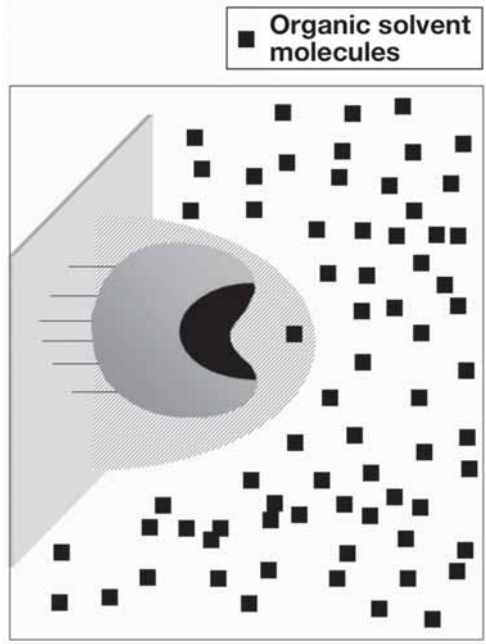

Fig. 7. Generation of hyperhydrophilic environments surrounding immobilized enzymes

fication—via hydrophobic or electrostatic interactions—are also impossible. Modifications of immobilized enzymes producing very different protein surfaces, however, can be performed, (e.g., hydrophobization of enzyme surfaces, physical adsorption of hyperhydrophilic polymers, or chemical cross-linking of surface residues).

As a consequence of chemical modification, the conformational changes involved in inactivation or catalysis of immobilized enzymes can be strongly modified. In this way, some improvements in stability or selectivity may be observed.

4.6. Generation of Hyperhydrophilic Microenvironments Surrounding Every Molecule of Immobilized Derivatives

Coimmobilization of enzymes and very high concentrations of very high-molecular-weight (MW) hydrophilic polymers can promote the generation of new hyperhydrophilic microenvironments that surround every molecule of immobilized enzyme (*see* **Fig. 7**). These microenvironments can promote partition phenomena of hydrophobic substances (e.g., cosolvents, substrates, products) between the bulk solution and the environment where every molecule of the immobilized enzyme is placed. Again, stability and catalytic behavior of these immobilized enzymes can become strongly modified and improved ***(28–30)***.

5. Immobilization of the Enzymes, Reaction Media, Reactor, and Application of the Immobilized Derivative

Obviously, the preparation of suitable immobilized enzyme derivatives strongly depends on the further utilization of such derivatives.

5.1. The Reaction Medium

Enzyme biotransformations can be carried out in very different reaction media such as aqueous media, anhydrous organic solvents, supercritical fluid, and ionic liquids. The supports and the immobilization protocols are strongly dependent on the reaction medium in which the immobilized enzyme is going to be used or tested.

5.2. The Reactors

Enzyme biotransformations can be carried out in very different types of reactors, such as stirred tanks, packed beads, fluidized beds, and basket reactors. The mechanics and hydrodynamics of immobilized derivatives and supports are strongly related to the reactor to be used.

5.3. Applications

Immobilized enzymes are highly versatile may be used in many applications, such as chemical reactors for fine chemistry, repeated reaction cycles in food chemistry, different types of devices (for one or many reaction cycles) in analytical chemistry, and therapeutic applications (e.g., inside humans for long periods of time). The supports, immobilization protocols, and properties of immobilized derivatives will be very dependent on the particular application.

6. Basic Applications of Immobilized Enzymes and Proteins

Immobilization of enzymes and other proteins may also be useful at the laboratory scale for the evaluation of critical properties of enzymes and proteins (e.g., assembly of multimeric enzymes, studies of unfolding and refolding of proteins, studies of enzymes attached to cofactors, studies on protein–protein interactions). The full retention of all structural and functional properties of immobilized enzymes, even the most negative, has to be critical. In this way, immobilization protocols have to cover quite a range of structural and functional necessities ranging from unaltered immobilized enzymes (*see* **Fig. 8**) to those that have been greatly modified and improved ***(10,31)***.

7. Immobilization of Other Biomacromolecules

Different protocols for enzyme immobilization can be useful for the immobilization of other biomolecules such as antibodies ***(32,33)*** and DNA probes ***(34,35)***. On one hand, antibodies can be immobilized on different supports (pre-existing porous supports, magnetic nanoparticles) with a correct orientation through their glycosylated chains of their Fc regions ***(32,33)***. This immobilization is similar to the immobilization of other glycosylated enzymes. On the other hand, DNA probes (i.e., those containing amino or thiol groups inserted in one terminus) could also be covalently immobilized (*see* **Subheading 6.**) for pure immobilization of enzymes. In both cases, the final surface of the support should be fully inert in order to prevent undesirable adsorption of antigens or DNA ***(35,37)***. These "tailor-made" immobilizations may be critical in very recent studies in genomics (e.g., molecular hybridization of DNA under experimental conditions favoring the "perfect match") and proteomics (e.g., interaction of antibodies with traces of antigens and protein–protein interactions).

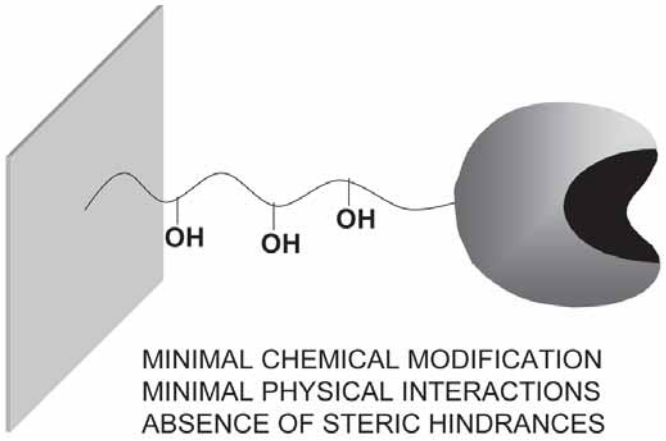

Fig. 8. Immobilization of an enzyme without altering its properties.

8. Enzyme Immobilization: Still a Fascinating Challenge

Thousands of protocols for enzyme immobilization have been reported over the past 30 yr. On their surfaces, enzymes have very different structural moieties that are able to interact (i.e., adsorption/covalent attachment) with such activated supports as nucleophilic residues, hydrophobic pockets, carboxylic groups, regions with net-positive charge, and regions with net-negative charge. In a certain way, and some authors maintain this point of view, it can be assumed that the problem of enzyme immobilization has already been solved. However, very simple and very efficient protocols (in terms of activity, stability, selectivity, and absence of inhibitions) for enzyme immobilization have not been fully developed. Therefore, enzyme immobilization must still be considered a fascinating challenge in modern biotechnology—much hard and interdisciplinary work remains. The improvement of protocols for enzyme immobilization should encompass molecular biology, protein chemistry, material science, and chemical engineering, among others. Very simple and efficient protocols for enzyme immobilization would greatly improve the possibilities for a massive implementation of industrial enzymes. A more ambitious and sustainable chemistry could be developed (e.g., the synthesis, under very mild experimental and environmental conditions, of very complex, cost-effective, and useful bioactive molecules).

9. The Immobilization of Enzymes in this Volume

This second edition of *Immobilization of Enzymes and Cells* aims to update and complement the first edition. This contributor will show some well-developed protocols for enzyme immobilization in order to encourage the development of new

and more efficient immobilization methods. Protocols to improve immobilization and use of immobilized derivatives have been presented according to the following criteria:

1. Very simple, ready for industrial application, protocols for enzyme immobilization.
2. Very efficient protocols that allow for great improvement of the enzyme properties as a consequence of immobilization.
3. Novel protocols for enzyme immobilization that could be very useful in the future.
4. Protocols for characterization of immobilized enzymes that are useful for the improvement of immobilization methods.
5. New reactors for improving the utilization of immobilized derivatives.
6. Protocols for immobilization directed to different uses and applications of industrial enzymes.

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2

Immobilization of Enzymes

A Literature Survey

Beatriz M. Brena and Francisco Batista-Viera

Summary

The term "immobilized enzymes" refers to "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously.". Besides the application in industrial processes, the immobilization techniques are the basis for making a number of biotechnological products with applications in diagnostics, bioaffinity chromatography, and biosensors. Initially, only immobilized single enzymes were used, but the 1970s saw the development of more complex systems—including two-enzyme reactions with co-factor regeneration and living cells. The major components of an immobilized enzyme system are the enzyme, the matrix, and the mode of attachment. The enzymes can be attached to the support by interactions ranging from reversible physical adsorption and ionic linkages to stable covalent bonds. The covalent reactions commonly employed give rise to binding through amide, ether, thio-ether, or carbamate bonds. As a consequence of enzyme immobilization, some properties such as catalytic activity or thermal stability become altered. These effects have been demonstrated and exploited. The concept of stabilization has been an important driving force for immobilizing enzymes. True stabilization at the molecular level has been demonstrated (e.g., proteins immobilized through multipoint covalent binding).

Key Words: Bioaffinity chromatography; biosensors; enzyme stabilization; immobilization methods; immobilized enzymes.

1. Background

Enzymes are biological catalysts that promote the transformation of chemical species in living systems. These molecules, consisting of thousands of atoms in precise arrangements, are able to catalyze the multitude of different chemical

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reactions occurring in biological cells. Their role in biological processes and in health and disease has been extensively investigated. They have also been a key component in many ancient human activities, especially food processing, well before their nature or function was known ***(1)***.

Enzymes have the ability to catalyze reactions under very mild conditions with a very high degree of substrate specificity, thus decreasing the formation of by-products. Among the reactions catalyzed are a number of very complex chemical transformations between biological macromolecules, which are not accessible to ordinary methods of organic chemistry. This makes them very interesting for biotechnological use. At the beginning of the 20th century, enzymes were shown to be responsible for fermentation processes and their structure and chemical composition started to come under scrutiny ***(2)***. The resulting knowledge led to widespread technological use of biological catalysts in a variety of other fields such as the textile, pharmaceutical, and chemical industries. However, most enzymes are relatively unstable, their costs of isolation are still high, and it is technically very difficult to recover the active enzyme, when used in solution, from the reaction mixture after use.

Enzymes can catalyze reactions in different states: as individual molecules in solution, in aggregates with other entities, and as attached to surfaces. The attached—or "immobilized"—state has been of particular interest to those wishing to exploit enzymes for technical purposes. The term "immobilized enzymes" refers to "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously" ***(3)***. The introduction of immobilized catalysts has, in some cases, greatly improved both the technical performance of the industrial processes and their economy (**Table 1**).

The first industrial use of immobilized enzymes was reported in 1967 by Chibata and co-workers, who developed the immobilization of *Aspergillus oryzae* aminoacylase for the resolution of synthetic racemic D-L amino acids ***(4)***. Other major applications of immobilized enzymes are the industrial production of sugars, amino acids, and pharmaceuticals ***(5)*** (**Table 2**). In some industrial processes, whole microbial cells containing the desired enzyme are immobilized and used as catalysts ***(6)***.

Aside from the application in industrial processes, the immobilization techniques are the basis for making a number of biotechnological products with applications in diagnostics, bioaffinity chromatography, and biosensors ***(7,8)***. Therapeutic applications are also foreseen, such as the use of enzymes in extra–corporeal shunts ***(9)***.

In the past three or four decades, immobilization technology has developed rapidly and has increasingly become a matter of rational design; but there is still the need for further development ***(10)***. Extension of the use of immobilized enzymes to other practical processes will require new methodologies and a better understanding of current techniques.

Table 1
Technological Properties of Immobilized Enzyme Systems *(3)*

Advantages	*Disadvantages*
Catalyst reuse	Loss or reduction in activity
Easier reactor operation	Diffusional limitation
Easier product separation	Additional cost
Wider choice of reactor	

Table 2
Major Products Obtained Using Immobilized Enzymes *(3)*

Enzyme	Product
Glucose isomerase	High-fructose corn syrup
Amino acid acylase	Amino acid production
Penicillin acylase	Semi-synthetic penicillins
Nitrile hydratase	Acrylamide
β-Galactosidase	Hydrolyzed lactose (whey)

2. History of Enzyme Immobilization

It is possible to visualize three steps in the development of immobilized biotatalysts (**Table 3**). In the first step, at the beginning of the 19th century, immobilized microorganisms were being employed industrially on an empirical basis. This was the case with both the microbial production of vinegar (by letting alcohol-containing solution trickle over wood shavings overgrown with bacteria) and the development of the trickling filter—or percolating process—for waste water clarification ***(11)***.

The modern history of enzyme immobilization goes back to the late 1940s but much of the early work was largely ignored by biochemists because it was primarily published in journals of other disciplines ***(12)***. The basis of the present technologies was developed in the 1960s and there was an explosive increase in publications. ***(4)***. In the second step, only immobilized single enzymes were used but by the 1970s more complex systems, including two-enzyme reactions with co-factor regeneration and living cells, were developed ***(13)***. As an example of the latter we can mention the production L-aminoacids from α-keto acids by stereoselective reductive amination with L-aminoacid dehydrogenase. The process involves the consume of nicotinamide adenine dinucleotide (NADH) and regeneration of the coenzyme by coupling the amination with the enzymatic oxidation of formic acid to carbon dioxide with concomitant reduction of NAD^+ to NADH, in the reaction catalyzed by the second enzyme, formate dehydrogenase.

Table 3
Steps in the Development of Immobilized Enzymes *(11)*

Step	Date	Use
First	1815	Empirical use in processes such as acetic acid and waste water treatment.
Second	1960s	Single enzyme immobilization: production of L-aminoacids, isomerization of glucose.
Third	1985–1995	Multiple-enzyme immobilization including co-factor regeneration and cell immobilization. Example: production of L-aminoacids from keto-acids in membrane reactors.

The major components of an immobilized enzyme system are the enzyme, the matrix, and the mode of attachment of the enzyme to the matrix. The terms solid-phase support, carrier, and matrix are used synonymously.

3. Choice of Supports

The characteristics of the matrix are of paramount importance in determining the performance of the immobilized enzyme system. Ideal support properties include physical resistance to compression, hydrophilicity, inertness toward enzymes ease of derivatization, biocompatibility, resistance to microbial attack, and availability at low cost ***(12–14)***.

Supports can be classified as inorganic and organic according to their chemical composition (**Table 4**). The organic supports can be subdivided into natural and synthetic polymers ***(15)***.

The physical characteristics of the matrices (such as mean particle diameter, swelling behavior, mechanical strength, and compression behavior) will be of major importance for the performance of the immobilized systems and will determine the type of reactor used under technical conditions (i.e., stirred tank, fluidized, fixed beds). In particular, pore parameters and particle size determine the total surface area and thus critically affect the capacity for binding of enzymes. Nonporous supports show few diffusional limitations but have a low loading capacity. Therefore, porous supports are generally preferred because the high surface area allows for a higher enzyme loading and the immobilized enzyme receieves greater protection from the environment. Porous supports should have a controlled pore distribution in order to optimize capacity and flow properties. In spite of the many advantages of inorganic carriers (e.g., high stability against physical, chemical, and microbial degradation), most of the industrial applications are performed with organic matrices. The hydrophilic character is one of the most important factors determining the level of activity of an immobilized enzyme ***(16)***.

An excellent matrix that has been extensively used is agarose. In addition to its high porosity, which leads to a high capacity for proteins, some other advantages of using agarose as a matrix are hydrophilic character, ease of derivatization, ab-

Table 4
Classification of Supports

Organic
Natural polymers • Polysaccharides: cellulose, dextrans, agar, agarose, chitin, alginate • Proteins: collagen, albumin • Carbon Synthetic polymers • Polystyrene • Other polymers: polyacrylate polymethacrylates, polyacrylamide, polyamides, vinyl, and allyl-polymers
Inorganic
Natural minerals: bentonite, silica Processed materials: glass (nonporous and controlled pore), metals, controlled pore metal oxides

sence of charged groups (which prevents nonspecific adsorption of substrate and products), and commercial availability. However, an important limitation in the use of agarose and other porous supports is the high cost. This problem can be circumvented by employing reversible methods that allow matrix regeneration and re-use.

The enzymes can be attached to the support via interactions ranging from reversible physical adsorption and ionic linkages to stable covalent bonds. One way of classifying the various approaches to immobilizing enzymes is in two broad categories: irreversible and reversible methods ***(17)***. The strength of the binding is usually inversely related to the ease with which it can be reversed. These two conflicting objectives—stability and reversibility—are difficult to fulfill simultaneously. The traditional approach has been to make the bond as strong as possible and sacrifice reversibility.

4. Methods of Irreversible Enzyme Immobilization

The concept of irreversible immobilization means that once the biocatalyst is attached to the support it cannot be detached without destroying either the biological activity of the enzyme or the support. The most common procedures of irreversible enzyme immobilization are covalent coupling, entrapment or micro-encapsulation, and cross-linking (*see* **Fig. 1**).

4.1. Formation of Covalent Bonds

Immobilization of proteins by methods based on the formation of covalent bonds are among the most widely used. An advantage of these methods is that, because of the stable nature of the bonds formed between enzyme and matrix, the enzyme is not released into the solution upon use. However, in order to achieve

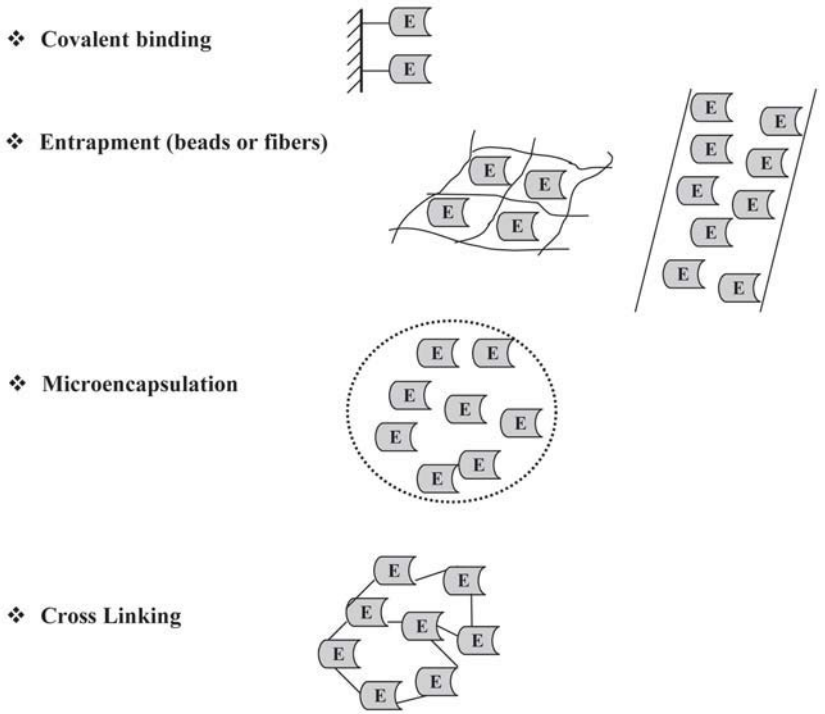

Fig. 1. Approaches to enzyme immobilization, irreversible methods.

high levels of bound activity, the amino acid residues essential for catalytic activity must not be involved in the covalent linkage to the support; this may prove a difficult requirement to fulfill in some cases. A simple procedure that sometimes improves the activity yield is to carry out the coupling reaction in the presence of substrate analogs ***(18)***. Covalent methods for immobilization are employed when there is a strict requirement for the absence of the enzyme in the product.

A wide variety of reactions have been developed depending on the functional groups available on the matrix ***(19)***. Coupling methods in general can be divided in two main classes: (1) activation of the matrix by addition of a reactive function to a polymer and (2) modification of the polymer backbone to produce an activated group (**Table 5**). The activation processes are generally designed to generate electrophilic groups on the support which, in the coupling step, react with the strong nucleophiles on the proteins. The basic principles controlling the course of covalent coupling to the matrices are analogous to those used for the chemical modification of proteins. The most frequently used reactions involve the following side chains of the amino acids: lysine (ε-amino group), cysteine (thiol group), and aspartic and glutamic acids (carboxylic group).

Table 5A
Covalent Coupling Methods of Enzymes: Activation of Matrix Hydroxyl Functions

Activation method	Group that reacts (with activated matrix)	References
Tresyl chloride, sulfonyl chloride	Thiol, amines	***22***
Cyanogen bromide	Amine	***23***
Bisoxiranes (epoxides)	Thiol, amine	***24***
Epichlorohydrin	Thiol, amine	***24***
Glutaraaldehyde	Amine	***24***
Glycidol-Glyoxyl	Amine	***25***
N-Hydroxy-succinimidyl	Amine	***26,27***

There are many commercially available supports for immobilization, the best choice in each case requires the consideration of some relevant properties of the catalyst and the intended use. However, it is usually necessary to try more than one approach and then adapt a method to the specific circumstances ***(20,21)***.

The covalent reactions commonly employed give rise to enzymes linked to the support through either amide, ether, thio-ether, or carbamate bonds. Therefore, the enzyme is strongly bound to the matrix and, in many cases, it is also stabilized, which will be discussed later in **Subheading 6**. However, because of the covalent nature of the bond, the matrix has to be discarded together with the enzyme once the enzymatic activity decays. The benefit of obtaining a leak-proof binding between enzyme and matrix resulting from these reactions is partially offset by the cost, in terms of generally low yield of immobilized activity and by the nonreversible character of this binding. Enzymes attached covalently by disulfide bonds to solid supports represent one way to avoid this problem and will be described in Chapter 17.

4.2. Entrapment

The entrapment method is based on the occlusion of an enzyme within a polymeric network that allows the substrate and products to pass through but retains the enzyme ***(35)***. This method differs from the coupling methods described above, in that the enzyme is not bound to the matrix or membrane. There are different approaches to entrapping enzymes such as gel ***(36)*** or fiber entrapping ***(37)*** and micro-encapsulation ***(38)***. The practical use of these methods is limited by mass transfer limitations through membranes or gels.

5. Methods of Reversible Immobilization

Because of the type of the enzyme-support binding, reversibly immobilized enzymes can be detached from the support under gentle conditions (*see* **Fig. 2**). The use of reversible methods for enzyme immobilization is highly attractive, mostly for economic reasons because when the enzymatic activity decays the support can be regenerated and re-loaded with fresh enzyme. Indeed, the cost of the

Table 5B
Modification of the Polymer Backbone to Produce an Activated Group

Polymer	Group that reacts	Reagent	Activated group produced	Group that reacts (with activated matrix)	Refs.
Cellulose agarose	Diol	Periodate	Aldehyde	Amine	*28*
Polyacrylamide	Amide	Hydrazine	Hydrazide	Amine	*29*
Polyacrylamide	Amide	Acid pH	Carboxylic acid	Amine	*29*
Polyester	Ester	Acid pH	Carboxylic acid + alcohol	Amine	*30*
Polyethylene	CH_2	Conc. Nitric acid	Carboxylic acid	Amine	*31*
Polystyrene		Conc. Nitric acid	Nitrated aromatic ring	Histidine, Tyrosine	*32,33*
Nylon	Amide	Hydrazine	Hydrazide	Amine	*34*

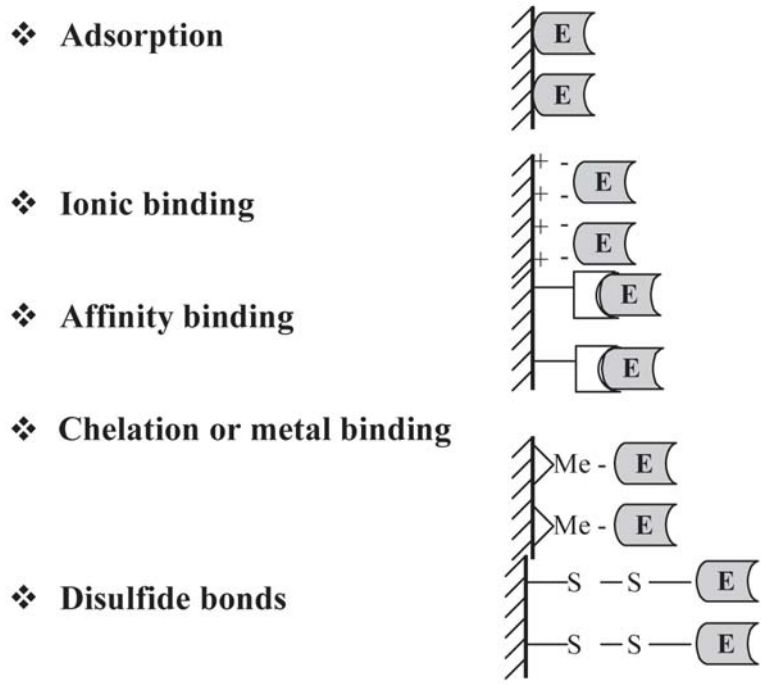

Fig. 2. Approaches to enzyme immobilization, reversible methods.

support is often a primary factor in the overall cost of immobilized catalyst. The reversible immobilization of enzymes is particularly important for immobilizing labile enzymes and for applications in bioanalytical systems ***(17)***.

5.1. Adsorption (Noncovalent Interactions)

5.1.1. Nonspecific Adsorption

The simplest immobilization method is nonspecific adsorption, which is mainly based on physical adsorption or ionic binding ***(39,40)***. In physical adsorption the enzymes are attached to the matrix through hydrogen bonding, van der Waals forces, or hydrophobic interactions; whereas in ionic bonding the enzymes are bound through salt linkages. The nature of the forces involved in noncovalent immobilization results in a process can be reversed by changing the conditions that influence the strength of the interaction (e.g., pH, ionic strength, temperature, or polarity of the solvent). Immobilization by adsorption is a mild, easy to perform process, and usually preserves the catalytic activity of the enzyme. Such methods are therefore economically attractive, but may suffer from problems such as enzyme leakage from matrix when the interactions are relatively weak.

5.1.2. Ionic Binding

An obvious approach to the reversible immobilization of enzymes is to base the protein–ligand interactions on principles used in chromatography. For example, one of the first applications of chromatographic principles in the reversible immobilization of enzymes was the use of ion-exchangers ***(4,41,42)***. The method is simple and reversible but, in general, it is difficult to find conditions under which the enzyme remains both strongly bound and fully active. More recently, the use of immobilized polymeric-ionic ligands has allowed for modulation of protein–matrix interactions and has thus optimized the properties of the derivative. A number of patents have been filed on the use of polyethyleneimine to bind a rich variety of enzymes and whole cells ***(43)***.

However, problems may arise from the use of a highly charged support when the substrates or products themselves are charged; the kinetics are distorted as a result of partition or diffusion phenomena. Therefore, enzyme properties, such as pH optimum or pH stability, may change ***(44,45)***. Although this could pose a problem it could also be useful to shift the optimal conditions of a certain enzyme towards more alkaline or acidic conditions, depending on the application ***(46)***.

5.1.3. Hydrophobic Adsorption

Another approach is the use of hydrophobic interactions. In this method, it is not the formation of chemical bonds but rather an entropically driven interaction that takes place. Hydrophobic adsorption has been used as a chromatographic principle for more than three decades. It relies on well-known experimental variables such as pH, salt concentration, and temperature ***(47)***. The strength of interaction relies on both the hydrophobicity of the adsorbent and the protein. The hydrophobicity of the adsorbent can be regulated by the degree of substitution of the support and by the size of the hydrophobic ligand molecule. The successful reversible immobilization of β-amylase and amyloglucosidase to hexyl-agarose carriers has been reported ***(48,49)***. Several other examples of strong reversible binding to hydrophobic adsorbents have also been reported ***(50–52)***.

5.1.4. Affinity Binding

The principle of affinity between complementary biomolecules has been applied to enzyme immobilization. The remarkable selectivity of the interaction is a major benefit of the method. However, the procedure often requires the covalent binding of a costly affinity ligand (e.s., antibody,= or lectin) to the matrix ***(53)***.

5.2. Chelation or Metal Binding

Transition metal salts or hydroxides deposited on the surface of organic carriers become bound by coordination with nucleophilic groups on the matrix. Mainly titanium and zirconium salts have been used and the method is known as “metal link immobilization” ***(15,54,55)***. The metal salt or hydroxide is precipitated onto the support (e.g., cellulose, chitin, alginic acid, and silica-based carriers) by heating or neutralization. Because of steric factors, it is impossible for the matrix to occupy all coordination positions of the metal; therefore some of the positions remain free to coordinate with groups from the enzymes. The method is quite simple and the immobilized specific activities obtained with enzymes in this way

have been relatively high (30–80%) However, the operational stabilities achieved are highly variable and the results are not easily reproducible. The reason for this lack of reproducibility is probably related to the existence of nonuniform adsorption sites and to a significant metal ion leakage from the support. In order to improve the control of the formation of the adsorption sites, chelator ligands can be immobilized on the solid supports by means of stable covalent bonds. The metal ions are then bound by coordination and the stable complexes formed can be used for the retention of proteins. Elution of the bound proteins can be easily achieved by competition with soluble ligands or by decreasing pH. The support is subsequently regenerated by washing with a strong chelator such as ethylene diamine tetraacetic acid (EDTA) when desired. These metal chelated supports were named IMA Immobilized Metal-Ion Affinity (IMA) adsorbents and have been used extensively in protein chromatography ***(56,57)***. The approach of using different IMA-gels as supports for enzyme immobilization has been studied using *Eschericia coli* β-galactosidase as a model ***(58)***.

5.3. Formation of Disulfide Bonds

These methods are unique because, even though a stable covalent bond is formed between matrix and enzyme, it can be broken by reaction with a suitable agent such as dithiothreitol (DTT) under mild conditions. Additionally, because the reactivity of the thiol groups can be modulated via pH alteration, the activity yield of the methods involving disulfide bond formation is usually high—provided that an appropriate thiol-reactive adsorbent with high specificity is used ***(59)***. Immobilization methods based on this strategy are discussed in Chapter 17.

6. Properties of Immobilized Enzymes

As a consequence of enzyme immobilization, some properties of the enzyme molecule, such as its catalytic activity or thermal stability, become altered with respect to those of its soluble counterpart ***(11,60)***. This modification of the properties may be caused either by changes in the intrinsic activity of the immobilized enzyme or by the fact that the interaction between the immobilized enzyme and the substrate takes place in a microenvironment that is different from the bulk solution. The observed changes in the catalytic properties upon immobilization may also result from changes in the three-dimensional conformation of the protein provoked by the binding of the enzyme to the matrix. These effects have been demonstrated and, to a lesser extent, exploited for a limited number of enzyme systems.

Quite often when an enzyme is immobilized its operational stability is improved. The concept of stabilization has thus been an important driving force for immobilizing enzymes. In many cases, the observed operational stabilization is usually the result of loading an excess of enzyme, which in turn makes the process diffusion-controlled. However, true stabilization at the molecular level has also been demonstrated, such as the case of proteins immobilized through multipoint covalent binding ***(61)***. Studies carried out by several authors using different methods have demonstrated that there is a correlation between stabilization and the number of covalent bonds to the matrix ***(62–64)***. One of the main problems associated with the use of immobilized enzymes is the loss of catalytic activity, especially when the enzymes are acting on macromolecular substrates. Because of the limited

access of the substrate to the active site of the enzyme, the activity may be reduced to accessible surface groups of the substrate only. This steric restriction may, in turn, change the characteristic pattern of products derived from the macromolecular substrate ***(65)***. There are several strategies to avoid these steric problems such as selection of supports composed by networks of isolated macromolecular chains, careful choice of the enzyme residues involved in the immobilization, and use of hydrophilic and inert spacer arms ***(66)***.

7. The Biology of Enzyme Immobilization

Although the science of enzyme immobilization has developed as a consequence of its technical utility, one should recognize that the advantages of having enzymes attached to surfaces have been exploited by living cells for as long as life has existed. An inquiry into the biological role of enzyme immobilization may provide some lessons for biotechnologists and serve as a second point of departure, in addition to the purely chemical one. In fact, there is experimental evidence that the immobilized state might be the most common state for enzymes in their natural environment. The attachment of enzymes to the appropriate surface ensures that they stay at the site where their activity is required. This immobilization enhances the concentration at the proper location and it may also protect the enzyme from being destroyed. Multimolecular assembly depends normally on weak noncovalent forces and hydrophobic interactions, but sometimes on covalent bonds as well (e.g., disulphide bridges) ***(1,2)***. All these different forces have been exploited in the development of immobilized enzymes.

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3

Cross-Linked Enzyme Aggregates

Roger A. Sheldon, Rob Schoevaart, and Luuk M. van Langen

Summary

The economic viability of biocatalytic conversions is often dependent on finding an effective method for immobilization of the enzyme involved. This provides for its improved operational stability and facile recovery and re-use. Cross-linked enzyme aggregates (CLEAs®) constitute an effective methodology for enzyme immobilization with broad scope. The technique is exquisitely simple, involving precipitation from aqueous buffer and subsequent cross-linking of the resulting physical aggregates of enzyme molecules, and amenable to rapid optimization. The resulting CLEAs are stable, recyclable biocatalysts exhibiting high activity retention, in some cases higher than that of the free enzyme they were derived from. The enzyme does not need to be of high purity since the methodology essentially combines purification and immobilization into a single operation. The technique can also be applied to the preparation of combi-CLEAs containing two or more enzymes. For example, an oxynitrilase/nitrilase CLEA for the one-pot synthesis of (*S*) mandelic acid from benzaldehyde in high yield and enantioselectivity.

Key Words: Immobilized enzymes; cross-linked enzyme aggregates; CLEAs®; hyperactivation; lipase CLEAs; combi CLEAs; biocatalysis.

1. Introduction

Considerable effort has been devoted ***(1,2)*** to developing effective immobilization techniques to increase the operational stability of enzymes and to facilitate their recovery and recycling, thereby reducing the contribution of the enzyme to the cost of the final product. In some cases, immobilizing the enzyme can also lead to enhanced selectivity ***(3)***. Indeed, an effective method for enzyme immobilization is often essential for making a biotransformation commercially viable.

Immobilization methods can conveniently be divided into three types: binding to a support (carrier), encapsulation (e.g., in a polymeric gel), or via cross-linking techniques ***(4)***. The first method is the most common. It can involve physical adsorption on organic polymers like polypropylene or inorganic oxides, such as

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silica. A disadvantage of physical adsorption is that the enzyme is readily leached from the surface in an aqueous medium.

A distinct disadvantage of carrier-bound enzymes, whether they involve binding to or encapsulation in a carrier, is the dilution of catalytic activity resulting from the introduction of a large proportion of noncatalytic mass, generally ranging from 90 to >99% of the total mass. This inevitably leads to lower volumetric and space-time yields and lower catalyst productivities. Moreover, attempts to achieve high enzyme loadings usually lead to loss of activity. The third type of immobilization, via cross-linking of enzyme molecules with a bifunctional cross-linking agent, most commonly glutaraldehyde, does not suffer from this disadvantage. Because the molecular weight (MW) of the cross-linking agent is negligible compared with that of the enzyme the resulting biocatalyst essentially comprises 100% active enzyme.

1.1. Cross-Linking of Enzymes

The technique of protein cross-linking via the reaction of glutaraldehyde with reactive NH_2 groups on the protein surface was initially developed in the 1960s ***(5)***. However, this method of producing cross-linked enzymes (CLEs) had several drawbacks, such as low activity retention, poor reproducibility, low mechanical stability, and difficulties in handling the gelatinous CLEs.

Mechanical stability and ease of handling could be improved by cross-linking the enzyme in a gel matrix or on a carrier but this led to the disadvantageous dilution of activity just mentioned. Consequently, in the late 1960s, emphasis switched to carrier-bound enzymes, which became the most widely used method for enzyme immobilization for industrial use.

The cross-linking of a crystalline enzyme by glutaraldehyde was first described by Quiocho and Richards in 1964 ***(6)***. Their main objective was to stabilize enzyme crystals for X-ray diffraction studies, but they also showed that catalytic activity was retained. The use of cross-linked enzyme crystals (CLECs) as industrial biocatalysts was pioneered by scientists at Vertex Pharmaceuticals ***(7)*** and subsequently commercialized by Altus Biologics ***(8)***. The initial studies were performed with CLECs of thermolysin, of interest in the manufacture of aspartame, but the method was subsequently shown to be applicable to a broad range of enzymes ***(9–11)***.

CLECs proved significantly more stable to denaturation by heat, organic solvents, and proteolysis than the corresponding soluble enzyme or lyophilized (freeze-dried) powder. CLECs have been formulated as robust, highly active immobilized enzymes of controllable size, varying from 1 to 100 μm, for use in industrial biotransformations. Their operational stability and ease of recycling, coupled with their high catalyst and volumetric productivities, render them ideally suited for industrial application.

An inherent disadvantage of CLECs is the need to crystallize the enzyme, often a laborious procedure requiring enzyme of high purity. Hence, we reasoned that comparable results could possibly be achieved by simply precipitating the enzyme from aqueous solution, using standard techniques, and cross-linking the resulting physical aggregates of enzyme molecules. This indeed proved to be the case and led to the development of a new family of cross-linked enzymes, which we have called cross-linked enzyme aggregates (CLEA).

1.2. The CLEA Technology

It is well-known ***(12)*** that the addition of salts, organic solvents, or nonionic polymers to aqueous solutions of proteins leads to their precipitation as physical aggregates of protein molecules without perturbation of their tertiary structure, that is without denaturation. Indeed, aggregation induced by addition of ammonium sulfate, poly(ethyleneglycol), and some organic solvents is a commonly used method of protein purification.

These solid aggregates are held together by noncovalent bonding and readily collapse and redissolve when dispersed in water. We surmised that cross-linking of these physical aggregates would produce CLEAs in which the pre-organized superstructure of the aggregates, hence, their catalytic activity, would be maintained.

Initial experiments were performed ***(13)*** with penicillin G acylase (penicillin amidohydrolase, E.C. 3.5.1.11), an industrially important enzyme used in the synthesis of semi-synthetic penicillin and cephalosporin antibiotics ***(14)***. The free enzyme has limited thermal stability and a low tolerance to organic solvents, making it an ideal candidate for stabilization as a CLEA. Indeed, penicillin G acylase CLEAs, prepared by precipitation with, for example, ammonium sulfate or tert-butanol, proved to be effective catalysts for the synthesis of ampicillin ***(15)***. They exhibited activities comparable with those of CLECs of the same enzyme, with substantially less competing hydrolysis of the side chain. Analogous to the corresponding CLECs, the penicillin G acylase CLEAs also maintained their activity in organic solvents.

We then examined the effect of various parameters, such as the precipitant and the addition of additives such as surfactants and crown ethers, on the activities of CLEAs prepared from seven commercially available lipases ***(16)***. The activation of lipases by additives, such as surfactants, crown ethers and amines, is well documented and is generally attributed to the lipase being induced to adopt a more active conformation ***(17)***. We reasoned, therefore, that cross-linking of enzyme aggregates, resulting from precipitation in the presence of such an additive, would "lock" the enzyme in this more favorable conformation. Moreover, because the additive is not covalently bonded to the enzyme, the additive can subsequently be washed from the CLEA with, for example, an appropriate organic solvent.

Using this procedure we succeeded in preparing a variety of lipase CLEAs exhibiting levels of activity even higher than the corresponding free enzyme, that is, up to three times the hydrolytic activity and up to ten times the activity of the free enzyme in organic solvents ***(18)***. In addition, we also demonstrated that the experimental procedure for CLEA preparation could be further simplified by combining precipitation, in the presence or absence of additives, with cross-linking into a single operation.

Hence, the potential of the CLEA technology for preparing immobilized enzymes with high catalyst and volumetric activities, in some cases with activities significantly exceeding those of the native enzymes they were derived from, was firmly established ***(3)***. The method is exquisitely simple and can, in principle, be performed with relatively impure samples of enzymes.

Indeed, the methodology essentially combines purification of the enzyme (via precipitation) and immobilization into one step. It must be noted, however, that if

Table 1
Activity Recovery of a Variety of CLEAs

Enzyme	Activity recovery (%)
Penicillin acylase from *E. coli*	53
Penicillin acylase from *A. faecalis*	58
Lipase A from *C. antarctica*	263
Lipase B from *C. antarctica*	177
Lipase from *Thermomyces lanuginosa*	327
Lipase from *A. niger*	116
Hydroxynitrile lyase from *Prunus amygdalus*	72
Hydroxynitrile lyase from *Manihot esculenta*	110
Nitrilase from *Pseudomonas fluorescens*	50
Pyruvate decarboxylase from *Zymomonas mobilis*	90
Galactose oxidase from *Dactylium dendroides*	95
β-Galactosidase from *A. oryzae*	100

an impure sample containing a mixture of enzymes is used, this can lead to a CLEA containing more than one enzyme. We have used this to our advantage in the deliberate preparation of combi-CLEAs, containing two or more enzymes, for use in multistep, biocatalytic cascade processes, for example an oxynitrilase and a nitrilase.

1.3. Scope of CLEA Technology

Subsequent studies were aimed at demonstrating the applicability of the CLEA technology to the effective immobilization of a broad range of enzymes, including cofactor-dependent oxidoreductases and lyases, and the influence of the many parameters (temperature, pH, concentration, stirring rate, precipitant, and cross-linking agent) on the properties of the resulting CLEA. The relative simplicity of the operation ideally lends itself to high-throughput methodologies.

We have shown that, by a suitable optimization of the procedure, which may differ from one enzyme to another, the CLEA methodology is applicable to essentially any enzyme. We have prepared active and stable CLEAs from, in addition to penicillin G acylase and lipases, esterases, trypsin, oxynitrilases, nitrilases, galactosidase, an alcohol dehydrogenase, formate dehydrogenase, glucose oxidase, galactose oxidase, catalase, laccase, phytase, and pyruvate decarboxylase. **Table 1** gives examples of the activity retention observed with various enzymes.

Activity yields exceeding that of the native enzyme were first observed with lipases, but were subsequently also observed with other enzymes. **Table 2** gives some examples of activity recoveries obtained using a variety of precipitants with four different enzymes.

Various cross-linking agents are known and can be used. Although glutaraldehyde remains cheap and versatile, some enzymes are inactivated by this reagent. Most likely, active site residues are modified. To prevent the cross-linker from entering the active site or to prevent extreme conformational changes, we devel-

Table 2
Example of Typical Precipitants and Activity Recovery After Precipitation

Precipitant		CALB	Glc oxidase	β-galactosidase	Trypsin
1	buffer	100	100	100	100
2	methanol	64	0	0	89
3	ethanol	66	15	0	135
4	1-propanol	30	85	80	129
5	2-propanol	77	104	82	144
6	*t*-butanol	100	116	99	148
7	acetone	52	94	65	185
8	acetonitrile	75	116	88	151
9	DME	85	113	79	142
10	ethyllactate	39	127	82	142
11	ammonium sulfate	131	101	83	186
12	DMF	72	1	0	85
13	DMSO	107	2	0	131
14	PEG	138	114	100	153

oped a special cross-linker based on dextran (a mixture of glucose oligomers). The modified dextran is too large to enter the active site; hence, in those cases where glutaraldehyde caused loss of activity, active CLEAs were obtained using this large molecule as a cross-linking agent.

Because the enzyme molecules are packed together in a small volume as compared with the free protein in solution, one might expect mass-transport limitations with fast reactions. However, on the time scale of the average biocatalytic synthesis such effects generally turned out to be negligible.

One major advantage of CLEAs is their facile separation afterwards. Because their size is in the micrometer range, filtration is easier; brief centrifugation also results in complete recovery of the catalyst. The stability is highly dependent on the reaction conditions used, but typically detrimental conditions—such as high stirring rate or the bubbling of oxygen necessary for many oxidases—pose few problems. Furthermore, no foam formation is observed when using CLEAs.

1.4. Combi-CLEAs

As noted earlier, another advantage of the CLEA technology is that it provides the possibility of preparing immobilized biocatalysts containing more than one enzyme (combi-CLEAs). We have demonstrated this concept in the synthesis of (*S*)-mandelic acid, from benzaldehyde, and aqueous cyanide, using a combi-CLEA containing an (*S*)-oxynitrilase and a nitrilase ***(19)***. The use of a combination of an enantioselective oxynitrilase and an aselective nitrilase is that the latter catalyses hydrolysis of the intermediate cyanohydrin, thus driving the equilibrium of the first step to the right.

1.5. Structure of Cross-Linked Enzyme Aggregates

The number of enzyme molecules and the way they are packed together in an aggregate can be expected to have a crucial influence on the activity of the aggregate as a whole. Hence, knowing the influential factors and being able to control them would pave the way for changing the CLEA from a phenomenon into a mature, well-defined catalytic particle. During aggregation the solubility of the enzyme in the surrounding medium decreases. When this process is slow the enzyme might denature because of the severe force exerted on its structure. If it is able to find neighboring protein molecules to surround it in time, chances are fairly good that it will retain its tertiary structure. As seen from the high-throughput experiments, increasing the speed of aggregation in cases of poor activity recovery always gave a completely active aggregate. Now the question is, to what extent will enzymes aggregate? Most likely, the surface tension of the aggregate will set the diameter. It is similar to water/oil mixtures where small droplets (emulsion) are formed. Cell- or droplet-like structures of protein aggregates, depending on the surface hydrophobicity, can in that case be expected. The interplay between free-energy increases by interfacial surface formation and free-energy decrease in solids formation governs the critical nucleus size according to the classical nucleation theory. The final aggregate size of the primary particles is then governed by the ratio of nucleation and growth but will likely be small.

Scanning electron microscopy showed a very uniform structure of the aggregates. From the different enzyme aggregates we examined, two types emerged. Type 1 aggregate was formed by *Candida antarctica* lipase B (*see* **Fig. 1**). This enzyme is scarcely glycosylated and highly lipophilic. The diameter of the aggregate is about 1 μm with a small deviation. Taking an enzyme size for CaLB of $5 \times 5 \times 5$ nm, a single CLEA particle contains a maximum of 8×10^6 enzyme molecules. Aggregates built from enzymes with a more glycosylated surface or a surface with more hydrophilic residues were found to be smaller, approx 0.1 μm in diameter, called Type 2 aggregates. *C. rugosa* lipase (*see* **Fig. 2**) and *Prunus amygdalus R*-oxynitrilase are examples of that type. These enzymes are glycosylated and therefore have a more hydrophilic surface. One CLEA particle of this aggregate contains a maximum of 8×10^3 enzyme molecules. Although Type 1 aggregates can contain a thousand times more enzyme molecules than Type 2, in both cases the enzymes find themselves in an apparently ideal environment where their natural function is thriving. Because the enzyme molecules are packed together in a small volume compared to the free protein in solution, one might expect mass-transport limitations, particularly with fast reactions. If the CLEAs are finely dispersed in solution—as is the case when the cross-link step is completed and subsequently quenched—this effect is actually small.

1.6. Clustering of Aggregates

CLEAs can form larger clusters (*see* **Fig. 3**) that do have mass-transport limitations, especially in fast ultraviolet (UV) tests. The size of these clusters can be up to 100 μm, making them visible to the naked eye. The number of CLEAs in a cluster is much less uniform than enzymes in an aggregate: it can vary from a few to a few hundred thousand. The previous findings with laser scattering (*see* **Fig. 4**)

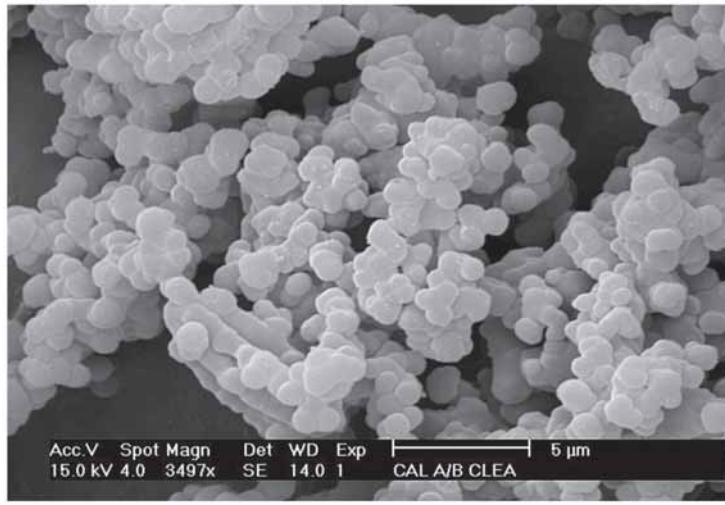

Fig. 1. *C. antarctica* lipase A/B CLEA. One CLEA particle can contain up to 8 million enzyme molecules (original magnification ×3500).

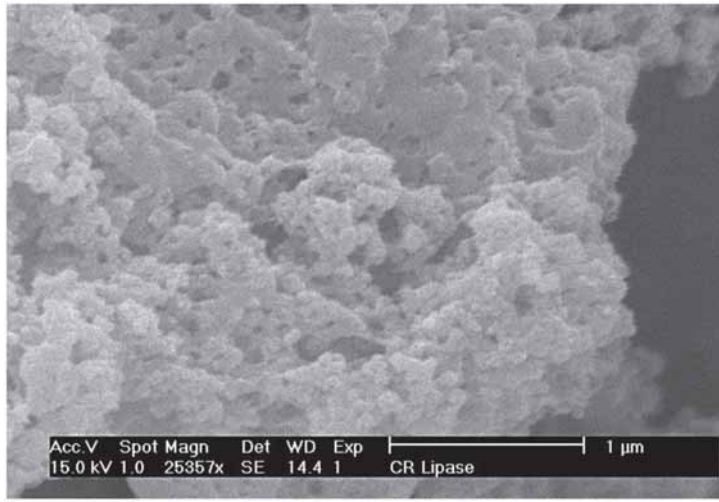

Fig. 2. *C. rugosa* lipase forms smaller CLEAs. One CLEA particle can contain up to 8000 enzyme molecules (magnification ×25000).

which suggested a variable number of enzyme molecules per aggregate is now rationalized as being a variable number of aggregates per cluster.

Centrifugation leads to increased cluster formation. When a dispersed CLEA is assayed for activity, directly after dilution of the cross-link medium, a higher

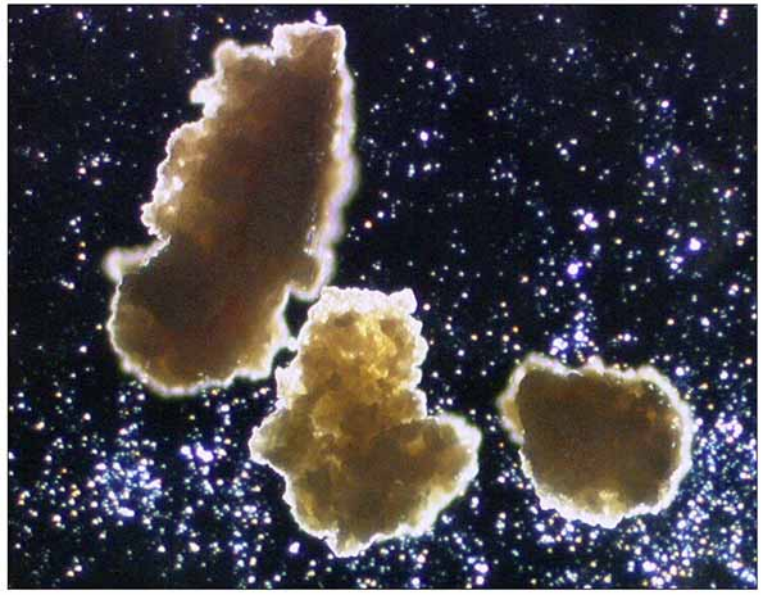

Fig. 3. *C. antarctica* lipase AB CLEA clusters in water (original magnification ×150).

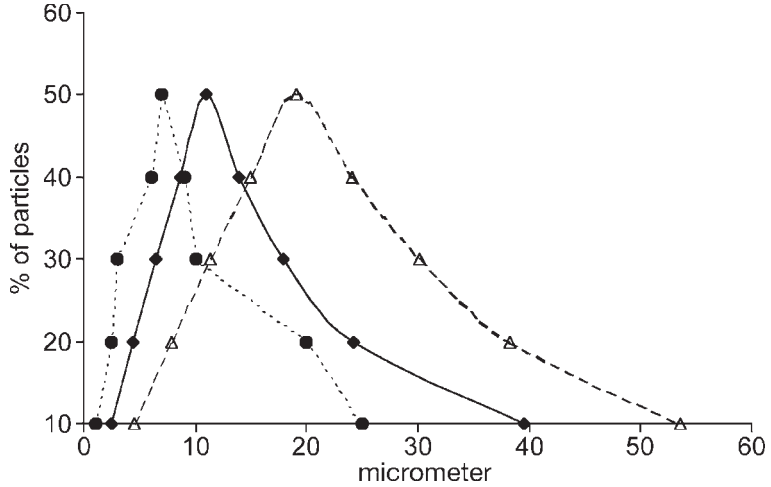

Fig. 4. CLEA particle-size analysis with laser scattering. Three different CLEA preparations are shown.

activity is observed than when the sample is centrifuged and redispersed. This presses the CLEA particles close together elevating mass-transport limitations to a noticeable level. The interaction between particles can be either reversible (as is demonstrated when these clusters are put in aqueous organic solvents, causing them to break up) or irreversible (when active cross-link residues on the CLEA surface form covalent bonds between individual particles). Some differences between enzymes were observed: with CaLB very large and hydrophobic clusters were obtained whereas with β-galactosidase well-dispersible suspensions were common. The most noticeable structural difference between these two enzymes is that β-galactosidase is extensively glycosylated and CaLB is not. Comparing activities for CaLB CLEA, found in the relatively fast hydrolysis of *p*-nitrophenyl propionate measured via UV/Vis absorption, with the slower hydrolysis of triacetin (monitored by titration) the mass-transport limitation was obvious. Compared with free enzyme the first showed 35% activity recovery and the latter 177%. For β-galactosidase however, activity recovery found in the hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside and in lactose hydrolysis was the same. These two CLEAs emphasize the important effect of cluster formation on the apparent activity.

1.7. Isolation of CLEAs

One major advantage of CLEAs is their facile separation from aqueous solutions. In contrast to free enzyme, brief centrifugation results in complete recovery of the catalyst. Another way of isolating CLEAs from a reaction mixture is filtration. The aggregate or cluster size determines how successful filtration will be. Certainly the large clusters do not pose any problem for even a large pore glass filter and the single sub-micrometer aggregates can be filtered with low-MW membrane filters. For medium size clusters the widely used micrometer pore size filters can be used. We applied several filters ranging from 0.2 to 5 μm. As expected, the 1 μm size *C. antarctica* lipase CLEAs passed through filters with pore sizes ranging from 1 to 5 μm. More surprisingly, they also passed through the 0.2 μm filters demonstrating the elasticity of the "enzyme balls." This suggests that the individual enzymes in an aggregate do have some freedom of movement which is undoubtedly beneficial for their activity. Smaller aggregates like the ones formed from *C. rugosa* lipase elegantly showed the effect of clustering. With their 0.1 μm size they could be filtered with 0.5 μm filters. Although the polypropylene (PPXL) filters were completely blocked, filtration was possible with the Zeta Plus filters which have a graded density resulting in better cake building. Controlling the size of the clusters means controlling the ability to filter the CLEAs.

1.8. Conclusions

The CLEA technology has many advantages in the context of industrial applications. The method is exquisitely simple and amenable to rapid optimization, which translates to low costs and short time-to-market. It is applicable to a wide variety of enzymes, including crude preparations, affording stable, recyclable catalysts with high retention of activity.

In contrast to CLECs, there is no need for the enzyme to be available in crystalline form and the technique can be applicable to the preparation of combi CLEAs containing two or more enzymes. Synthesis of CLEAs in the presence of addi-

tives, such as crown ethers or surfactants, provides the possibility of ”locking” the immobilized enzyme in a more favorable conformation, resulting in increased activity and/or (enantio)selectivities.

Currently the technology is being commercialized by CLEA Technologies on the basis of an exclusive licence from Delft University of Technology. We believe that CLEAs will, in the future, be widely applied in industrial biotransformations and other areas requiring immobilized enzymes.

2. Materials

1. Lipases (Novozymes, Bagsværd, Denmark).
2. Trypsin (Novozymes).
3. Galactose oxidase (Hercules, Barneveld, The Netherlands).
4. β-galactosidase and phytase (DSM, Delft, The Netherlands).
5. Laccase, alcohol dehydrogenase, and formate dehydrogenase (Jülich Fine Chemicals, Jülich, Germany).
6. All other enzymes and chemicals were purchased from Sigma.
7. Scanning Electron Microscope (Philips XL 20).
8. Samples of CLEAs were freeze-dried prior to analysis. UV/Vis spectroscopy was performed on a Varian Cary 3 Bio equipped with a Cary temperature controller or a Greiner microplate U-FORM 96K microtiter plate reader at room temperature.
9. 50-mL Centrifuge tube with a magnetic strirrer bar.
10. 25% (v/v) Glutaraldehyde solution.
11. Lipase assay buffer: 100 m*M* potassium phosphate, pH 7.4, 0.4 m*M* *p*-nitrophenyl propionate at 25°C.
12. Slow reaction substrate for lipases: Triacetin (2%v/v) in 50 m*M* Tris buffer, pH 7.4, at 40°C.
13. ABTS: 0.2 mg/mL0.1 *M* acetate, pH 4.5.
14. Phytase activity buffer: *p*-nitrophenyl phosphate (0.4 m*M*) in 0.1 *M* acetate, pH 4.5.
15. A solution containing 200 m*M* galactose (*see* **Note 1**), *o*-toluidine (1.75 m*M*), peroxidase (60 U/mL), and 100 m*M* potassium phosphate, pH 7.3, was used to detect galactose oxidase activity.
16. A solution containing glucose (200 m*M*) (*see* **Note 1**), *o*-toluidine (1.75 m*M*), peroxidase (60 U/mL), and 100 m*M* potassium phosphate, pH 7.3, was used to detect glucose oxidase activity.
17. β-Galactosidase activity buffer: 100 m*M* potassium phosphate, pH 7.3, 0.4 m*M* *p*-nitrophenyl-β-D-galactopyranoside with hydrolysis ***(24)*** was monitored at 25°C and 400 nm.
18. Alcohol dehydrogenase activity buffer: 1.5 m*M* *p*-chloro-acetophenone in 100 m*M* potassium phosphate buffer, pH 6.0, and 0.25 m*M* nicotinamide-adenine dinucleotide (NADH).
19. Formate dehydrogenase activity buffer: 100 m*M* potassium phosphate buffer, pH 7.5, containing 160 m*M* sodium formate and 0.25 m*M* NAD.
20. The filter system was supplied by CUNO Benelux. 0.2–5.0 µm PPXL filters and a 0.5 µm Zeta Plus 050 HT filter were used.

3. Methods

3.1. Cross-Linked Enzyme Aggregate Activity (see Note 2)

1. For the cross-link optimization 90 µL precipitant containing glutaraldehyde in varying concentrations was added to 10 µL enzyme solution (*see* **Note 3**).
2. The samples were incubated at room temperature without shaking. For the volumetric activity optimization 90 µL precipitant containing glutaraldehyde was added to 10 µL solution with a varying protein content and after the specific cross-linking time this mixture was quenched with 900 µL buffer.

3.2. General Cross-Linked Enzyme Aggregate Scale-Up

1. In a 50-mL centrifuge tube with a magnetic stirrer bar 1-mL enzyme (25 mg) solution in 100 m*M* buffer (mentioned in the activity assay) was added to 9-mL precipitant at room temperature unless stated otherwise.
2. Glutaraldehyde was then added to the desired end concentration and the suspension was stirred for 2.5 h.
3. The suspension was diluted with 10-mL buffer and centrifuged.
4. The pellet was resuspended in buffer and centrifuged again.
5. This procedure was performed two times in total.
6. Subsequently, the washed CLEA was resuspended in 1-mL buffer and stored at 4°C.

3.2.1. Lipase

1. The standard activity test was performed with *p*-nitrophenyl propionate as substrate (7.8 mg in 1 mL ethanol; 10 µL/mL lipase assay buffer).
2. The reaction was monitored at 400 nm (*see* **Note 4**).
3. Additionally, (slow) hydrolysis of 2% triacetine (v/v) in 50-m*M* Tris buffer, pH 7.4, and 40°C was performed by 0.1 *M* NaOH titration.
4. *C. antarctica* lipase A [EC 3.1.1.3] (CaLA) contained 125 U/mL.
5. The CLEA was prepared in DME with 100 m*M* glutaraldehyde and 7 U/mL.
6. *C. antarctica* lipase B [EC 3.1.1.3] (CaLB) contained 308 units and 10 mg protein/mL.
7. The Cal B CLEA was prepared in DME with 150 m*M* glutaraldehyde and 15 U/mL.
8. *Thermomyces lanuginosus* lipase [EC 3.1.1.3] (TlL) contained 66.7 U/mL.
9. The TlL CLEA was prepared in *tert*-butyl alcohol with 100 m*M* glutaraldehyde and 5 U/mL.
10. *Rhizomucor miehei* lipase [EC 3.1.1.3] (RmL) contained 44.1 U/mL.
11. The RmL CLEA was prepared in *tert*-butyl alcohol with 100 m*M* glutaraldehyde and 3 U/mL.

3.2.2. Laccase

1. Enzymatic oxidation ***(20)*** of ABTS was monitored at 420 nm and 25°C (*see* **Note 5**)
2. *Coriolus versicolor* laccase [EC 1.10.3.2] (Lacc) contained 0.66 U/mg.
3. The CLEA was prepared in polyethylene glycol (PEG) with 100 m*M* glutaraldehyde and 2 U/mL.

3.2.3. Phytase

1. Hydrolysis of *p*-nitrophenyl phosphate was monitored at 400 nm to determine the activities of phytase ***(21)***. (*See* **Note 6**).
2. *Aspergillus niger* phytase [EC 3.1.3.8] (Phyt) contained 47 U/mg.
3. The CLEA was prepared in ethyl lactate with 100 m*M* glutaraldehyde and 120 U/mL.

3.2.4. Galactose Oxidase

1. The oxidation of *o*-toluidine ***(22)*** was monitored at 425 nm.
2. *Dactylium dendroides* galactose oxidase [EC 1.1.3.9] (GalOx) contained 3000 U/mL.
3. The CLEA was prepared in *tert*-butyl alcohol with 100 m*M* glutaraldehyde and 225 U/mL.

3.2.5. Trypsin

1. The activity ***(23)*** was monitored using the same test as for the lipases (hydrolysis of *p*-nitrophenyl propionate).
2. Porcine Trypsin contained 30% protein/mg.
3. The CLEA was prepared in saturated ammonium sulphate with 100 m*M* glutaraldehyde and 2 mg/mL.

3.2.6. Glucose Oxidase

1. The oxidation of *o*-toluidine was monitored at 425 nm.
2. *A. niger* glucose oxidase [EC 1.1.3.4] (GlcOx) contained 50 U and 20% protein/mg.
3. The CLEA was prepared in saturated ethyl lactate with 100 m*M* glutaraldehyde and 125 U/mL.

3.2.7. β-Galactosidase

1. The hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside ***(24)*** is monitored at 25°C and 400 nm.
2. *A. oryzae* β-galactosidase [EC 3.2.1.23] (Gal-ase) contained 227 U/mg.
3. The CLEA was prepared in 2-propanol with 100 m*M* glutaraldehyde and 850 U/mL.

3.2.8. Alcohol Dehydrogenase

1. Enzyme activity ***(20)*** was monitored at 30°C and 340 nm (Mol ext. = 6.22 $mM^{-1}cm^{-1}$).
2. *Rhodococcus erythropolis* alcohol dehydrogenase [EC 1.1.1.2] (ADH) contained 77 U and 16.1 mg protein/mL.
3. The CLEA was prepared in saturated ammonium sulphate with 8 m*M* glutaraldehyde at 4°C and 7.7 U/mL.

3.2.9. Formate Dehydrogenase

1. Enzyme activity was detected ***(26)*** with an activity buffer at 30°C and 340 nm (Mol ext. = 6.22 $mM^{-1}cm^{-1}$).
2. *C. boidinii* formate dehydrogenase [EC 1.2.1.2] (**FDH**) contained 70 U and 26.3 mg/mL.
3. The CLEA was prepared in saturated ammonium sulphate with 8 m*M* glutaraldehyde at 4°C and 7 U/mL.

3.3. Scale-Up and Preparative Use of β-Galactosidase Cross-Linked Enzyme Aggregate

1. Add drop-wise 200 mg of β-galactosidase in 20-mL potassium phosphate buffer, pH 7.3, to 80 mL 2-propanol in a 250-mL flask with magnetic stirrer immersed in a water bath at room temperature.
2. After complete addition, 3.7 mL 25% glutaraldehyde was added and the suspension was stirred for 1 h.
3. CLEA was then centrifuged off, washed twice with 50 mL 50% ammonium sulphate solution, and subsequently stored in it.
4. To 20 mL of a 50 m*M* lactose solution in 25 m*M* potassium phosphate buffer, pH 7.3, either 4 mg of free enzyme was added or an equivalent CLEA suspension (made from 4 mg).
5. After 3 h or overnight incubation with magnetic stirring at room temperature the reaction mixtures were centrifuged and the supernatants concentrated *in vacuo*. ^{13}C NMR showed 55% conversion in both cases, whereas overnight incubation gave quantitative hydrolysis.
6. Upon assaying the recovered CLEA, it was found that no activity was lost during lactose hydrolysis.

3.4. Filtration

1. An amount of 100 mg CLEA was suspended in 10 mL of demineralized water. The liquid was pressed through the filter with 2 bar nitrogen gas.
2. The filtrate was then assayed for enzyme activity.

4. Notes

1. Solution is prepared 1 d in advance to allow for mutarotation.
2. For the assays, an enzyme activity is necessary that induces a change in absorption (ΔA) of 0.1 to 0.5/min. Dilutions of the enzymes were made so that the ΔA per minute never exceeded 0.5. All activities were correlated to the native enzyme, taken as 100% (no absolute activities are given).
3. To the glutaraldehyde stock 1 vol% phosphoric acid was added and the pH was adjusted to 7.3 with diluted sodium hydroxide prior to mixing with the precipitants
4. Blank reaction rate: ΔA 0.00318/min
5. The assay buffer was saturated with oxygen prior to addition to the cuvet.
6. The blank reaction at pH 4.5 was negligible.

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4

Immobilization–Stabilization of Enzymes by Multipoint Covalent Attachment on Supports Activated With Epoxy Groups

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Summary

Commercial epoxy supports may be very useful tools to stabilize proteins via multipoint covalent attachment if the immobilization is properly designed. In this chapter, a protocol to take full advantage of the support's possibilities is described. The basics of the protocol are as follows: (1) the enzymes are hydrophobically adsorbed on the supports at high ionic strength. (2) There is an "intermolecular" covalent reaction between the adsorbed protein and the supports. (3) The immobilized protein is incubated at alkaline pH to increase the multipoint covalent attachment, thereby stabilizing the enzyme. (4) The hydrophobic surface of the support is hydrophylized by reaction of the remaining groups with amino acids in order to reduce the unfavorable enzyme–support hydrophobic interactions. This strategy has produced a significant increase in the stability of penicillin G acylase compared with the stability achieved using conventional protocols.

Key Words: Multipoint covalent attachment; hydrophobic interactions; hydrophylization; enzyme stabilization.

1. Introduction

Many protocols for covalent immobilization of proteins have been reported. Many are very useful, at least to immobilize and solve the problem of the immobilization at laboratory scale. In these cases, the researcher has to be an expert in both support activation and protein immobilization methods ***(1–9)***. In fact, enzyme immobilization is already considered a very well-developed technique. However, most protocols have some drawbacks when used to quantitatively immobilize under mild experimental conditions. One is the need for large amounts of protein per milliliter of support throughout long-term handling of the activated supports when the immobilization is carried out at an industrial level. There are no

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current methodologies that may be used for immobilization of large amounts of enzyme in a solid under mild experimental conditions ***(1–9)***. Keeping in mind these considerations, epoxy-activated supports seem to be almost ideal systems with which to develop easy protocols for enzyme immobilization. Epoxy groups are very stable at neutral pH values so commercial supports can therefore be stored for long periods of time, meaning that they can be prepared in advance of the enzyme immobilization. Moreover, these epoxy supports may react with very different chemical groups present in the amino acids such as thiol, amine, or aromatic alcohols.

While other popular immobilization protocols may promote great alterations in the physical properties of the protein surface or in some instances yield labile enzyme–support attachments (e.g., when using BrCN-activated supports) ***(10,11)***, epoxy supports are able to react with different nucleophilic groups of the protein surface to form extremely strong linkages (secondary amino, ether, or thioether bonds) with a minimal chemical modification of the protein because pK values of the new secondary amino groups are very similar to those of the pre-existing primary amino ones. Thus, the linkages are established through short spacer arms. This is advantageous because they may be used to stabilize enzymes through multipoint covalent attachment via control of the enzyme–support interactions ***(12)***. In this way, as it is reported, the residues of proteins involved in immobilization retain their relative positions almost completely and are largely unaffected during any conformational change promoted by heat, pH, organic solvents, or any other distorting agents ***(13–15)***. Thus, such multipoint, covalently immobilized enzymes should remain more stable than both their soluble counterparts and other randomly immobilized preparations when exposed to any distorting reagent.

At the end of the immobilization process, epoxy groups can be easily blocked by reaction with very different thiol or amine compounds under mild conditions ***(16)***; this prevents further uncontrolled reaction between the support and the enzyme that might decrease its stability.

1.1. Enzyme Immobilization on Epoxy Supports

Negligible immobilizations are found when crude *Escherichia coli* protein preparations are incubated in the presence of epoxy–agarose supports at both high- and low-ionic strengths. Similar immobilization yields are found when other hydrophobic commercial supports are used at low ionic strength conditions. These results point out that the previous hydrophobic adsorption of proteins on these supports at high-ionic strength (described as necessary for the protein immobilization on these supports ***[12,17,18]***) is a consequence of the hydrophobicity of the support's core and not a result of the presence of the epoxy groups covering the support surface.

Thus, to immobilize proteins onto epoxy supports, the use of a hydrophobic support in the presence of high-ionic strength is necessary. In these conditions the previous adsorption of the protein at neutral pH (where the soluble enzymes are normally more stable) is promoted, and in a second step the covalent intramolecular reaction between the nucleophylic groups and the epoxide of the support is produced (*see* **Fig. 1**). In these conditions it is possible to immobilize 70% of the proteins contained in a crude strain of *E. coli*.

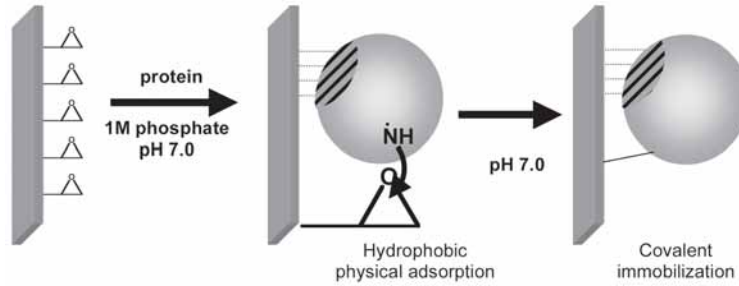

Fig. 1. Mechanism of immobilization of proteins on epoxy supports.

1.2. Optimization of Protein–Support Multipoint Covalent Attachment

As just mentioned, an intense multipoint covalent enzyme–support attachment can stabilize the protein structure, therefore highly stabilized enzyme preparations could be obtained.

The reactivity of the nucleophilic groups present in the protein surface becomes a key parameter in establishing a multipoint covalent attachment. These nucleophilic groups are poorly reactive at neutral pHs (*see* **Fig. 2**). Thus, multipoint covalent attachment requires long reaction times at alkaline pHs. Moreover, support and enzyme surfaces are not complementary; therefore, multipoint interaction may require long interaction times to permit the correct alignment between the reactive groups (*see* **Fig. 3**).

1.3. Blockage of the Remainder Epoxy Groups

The blockage of the immobilized support fulfills a double objective: (1) eliminate the reactivity of the support and (2) alter the physical properties of the support (important when considering their hydrophobic nature). A proper blockage may promote the hydrophylization of the support surface and avoid the hydrophobic interactions with the protein hydrophobic pockets that could interact with the partially distorted structure of the protein and promote an apparent "destabilization" of the enzyme (*see* **Fig. 4**).

In fact, most of the commercial supports recommend the use of 2-mercaptoethanol as a blocking agent. However, there are numerous compounds that are able to react with the epoxy groups—such as other thiols, amino acids, and other amines—that can yield hydrophilic surfaces.

2. Materials

1. Epoxy Sepabeads® EC-EP (Resindion SRL, Milan, Italy) (*see* **Note 1**).
2. Eupergit® C and 250L (Degussa, Dusseldorf, Germany) (*see* **Note 2**).
3. Immobilization buffer: 1 *M* sodium phosphate buffer, pH 7.0, adjusted with 5 *M* NaOH (*see* **Note 3**).
4. Incubation buffer: 100 m*M* sodium phosphate adjusted with 5 *M* NaOH to pH 10.0. Some additives may be used (*see* **Note 4**).
5. Blocking buffer: 3 *M* glycine, pH 8.5.

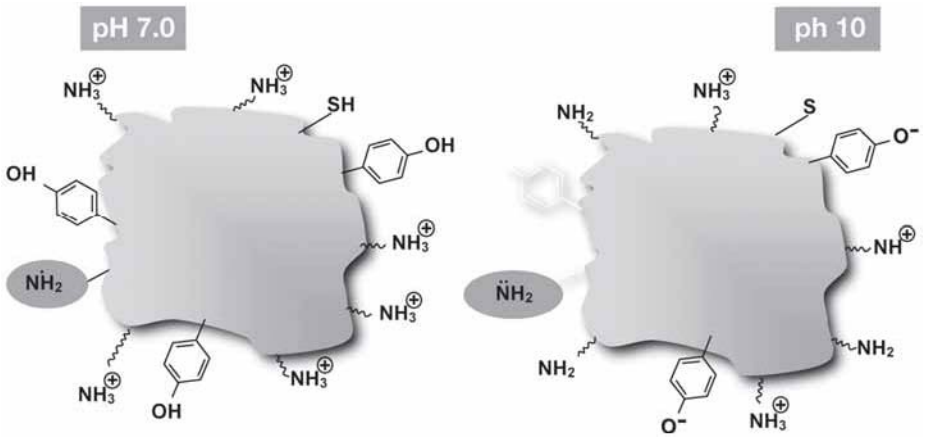

Fig. 2. Enzyme immobilization on epoxy supports at pH 7.0.

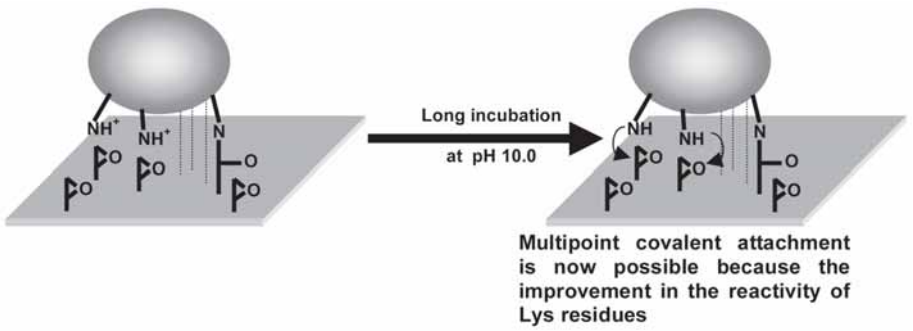

Fig. 3. Enzyme immobilization on epoxy supports at pH 10.0.

3. Methods

3.1. Preparation of Epoxy Supports and Enzyme Solution

1. Wash the support 10 times with 5 vol of distilled water, pH 7.0, at 4°C using a Büchner flask with a glass-sintered funnel connected to a vacuum line for filtration.
2. Dissolve the proteins in 1 *M* sodium phosphate, pH 7.0 (*see* **Subheading 2.**). Take a sample as a reference blank and test the enzyme activity (*see* **Note 5**).

3.2. Immobilization of Proteins on Amino-Epoxy Supports

1. Add the support to the enzyme solution.
2. Keep the suspension (enzyme and gel) under mild stirring at 25°C. (*see* **Note 6**)
3. Periodic samples of the supernatant and suspension were taken for assay of enzyme activity. Supernatant was achieved either by using pipet filter or by centrifugation of the suspension (*see* **Note 7**).

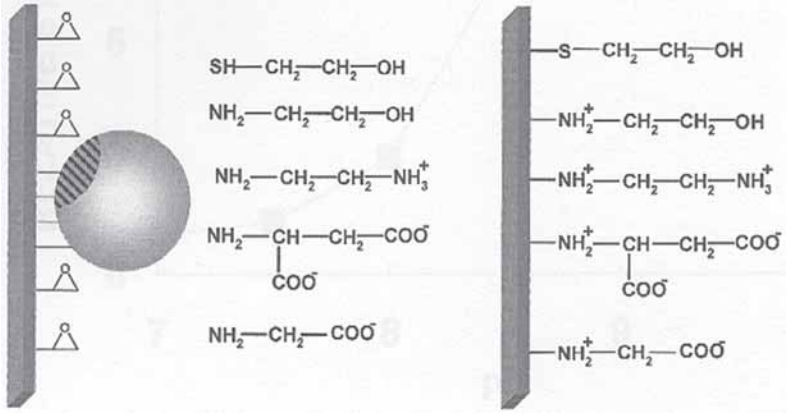

Fig. 4. Blockage of remainder epoxy groups with different compounds reactive with epoxy groups.

4. Assay the enzyme activity of the reference solution using the same time intervals as in **step 3**. The immobilization process is finished when the activity of the supernatant is zero.

3.3. Desorption of Proteins Noncovalently Immobilized on the Support

1. 2.5 mL Enzymatic suspension were taken and dried by filtration under vacuum filter.
2. The dried support was resuspended in 2.5 mL of distilled water. The suspension was left under mild stirring at 20°C for 30 min.
3. The enzyme activity or the protein concentration of the supernatant was checked.
4. If there are no proteins released, covalent attachment was considered.

3.4. Multi-Interaction Step on Enzyme Immobilization

1. The immobilized preparation was washed 5 times with 3 vol of 100 m*M* sodium phosphate buffer, pH 10.0, and then resuspended in 3 vol of that solution. Stirring is not necessary in this step.
2. The immobilized protein was left to interact with the support for varying times ranging from 1 d to 1 wk prior to incubation with 3 *M* glycine, pH 8.5. Activity of the immobilized preparations could be followed over the course of the incubation (e.g., every day) (*see* **Note 8**).

3.5. Blocking of Epoxy Groups of the Supports

1. The immobilized preparations were vacuum dried and resuspended in 3 vol 3 *M* glycine, pH 8.5, under gentle stirring for 8 h at 20°C.
2. Finally, the enzyme preparation was washed with distilled water and stored at 4°C.

3.6. Immobilization of Penicillin G-Acylase on Sepabeads EC-EP1

1. Prepare 90 mL of penicillin G-acylase solution (1 mg/mL) (Antibióticos S.A.) in 1 *M* sodium phosphate solution, pH 7.0, with 100 m*M* phenylacetic acid and 20% (v/v) glycerine (*see* **Note 4**).

2. Assay the catalytic activity of this solution. Add 10 g of Sepabeads EC-EP1 and assay the enzyme activity of both the suspension and supernatant at different immobilization times.
3. Gently stir the suspension for 24 h at 25°C.
4. After stirring, evaluate the covalent immobilization as described in **Subheading 3.3.**, **step 1.**
5. Incubate the enzyme suspension in 100 m*M* sodium phosphate buffer, pH 10, and gently stir the enzyme–support reaction for 5 d. The optimization process should be carried out as described in **Fig. 5A**,**B**. The stability was improved when the previously immobilized derivative was incubated at an increasing pH until an optimal pH of 9.0–10.0 was reached, with only a slight decrease in enzyme activity (*see* **Fig. 5A**). The stability also improved after incubation for extended periods of time, the optimal being 4-d incubation at pH 9.0 (*see* **Fig. 5B**).
6. Filter the suspension and then block the epoxy groups as described in **Subheading 3.5.**, **step 1**.
7. Wash and filter the suspension with 25 m*M* phosphate buffer, pH 7.0, and distilled water. Filter to eliminate inter-particle water.

3.7. Immobilization of Penicillin Acylase and Chymotrypsin on Eupergit C

1. Prepare 90 mL of chymotrypsin solution (1 mg/mL) (Sigma-Aldrich) or penicillin G acylase (Antibioticos) in 1 *M* sodium phosphate solution, pH 8.0.
2. Assay the catalytic activity of this solution. Add 10 g of Eupergit C and assay the enzyme activity of both the suspension and supernatant at different immobilization times.
3. Stir very gently for 24 h at 25°C.
4. After stirring, evaluate covalent immobilization as described in **Subheading 3.3.**
5. Incubate the enzyme suspension in 100 m*M* sodium bicarbonate buffer, pH 10.0, and gently stir the enzyme–support reaction for 5 d (*see* **Note 6**).
6. Filter the suspension and then block the epoxy groups as described in **Subheading 3.5.**, **step 1**.
7. Wash and filter the suspension with 25 m*M* phosphate buffer, pH 8.0, and distilled water as described in **Subheading 3.5.**, **step 2**.
8. Filter to dryness.

3.8. Thermal Stabilization of Chymotrypsin- and PGA-Eupergit C Derivatives (see *Table 1*)

1. Enzymes were immobilized as in **Subheading 3.7.**
2. Relative stability refers to the increase in the half life of the enzyme derivative as compared with the half life of the enzyme immobilized under standard conditions (pH 7.0 and 25°C).

4. Notes

1. Sepabeads EC-EP is a commercially available epoxy support (Resindion SRL, Milan, Italy), with different pore sizes: EC-EP1 (small) to EC-EP2 (medium), and finally EC-EP3 (large).

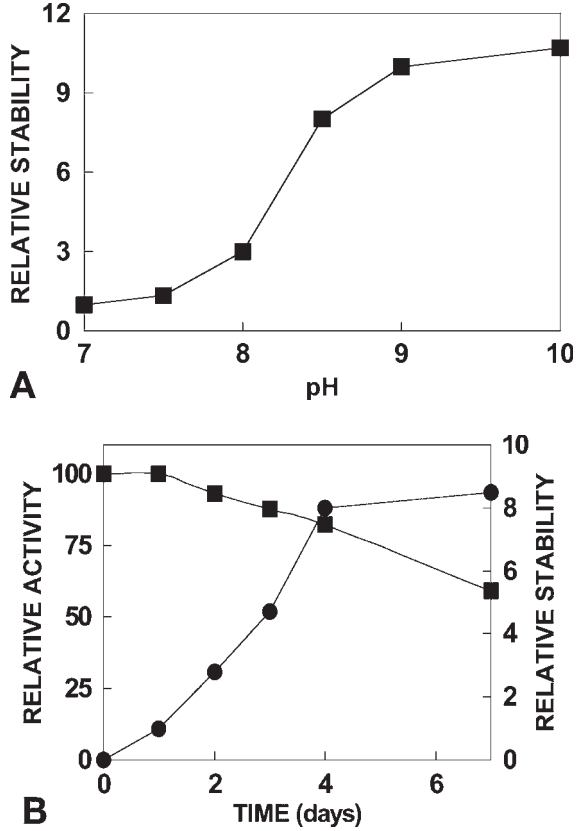

Fig. 5. (**A**) Effect of the pH of incubation on the stability of penicillin G acylase immobilized on Sepabeads supports. PGA was immobilized in 1 *M* sodium phosphate at pH 7.0 for 24 h. Afterwards, the pH was increased at the indicated pH value, and the suspension was incubated for 24 h before blocking of the epoxy groups with glycine as described in **Subheading 3.** (**B**) Effect of incubation time on the activity/stability of PGA immobilized on Sepabeads EC-EP1. PGA was immobilized at 1 *M* sodium phosphate at pH 7.0. After 24 h the pH of the immobilization was increased to 9.0 in the presence of phenyl acetic acid and glycerine. Finally, the immobilized preparations were blocked with 3 *M* glycine at the indicated times. (■) Residual activity; (●) relative stability. Relative stability is given as the ratio between the half-life of the problem preparation divided by the half-life of the preparation just immobilized at pH 7.0 and blocked.

2. Eupergit are oxirane–acrylic commercially available supports from Degussa with different average particle size and pore degrees ranging from C (little) to CM (medium) and finally C 250 L (large).
3. A high concentration of sodium phosphate buffer is required for physical hydrophobic adsorption onto the support.
4. Competitive inhibitor of the enzyme may be required to avoid enzyme inactivation or to preserve the enzyme structure.

Table 1
Effect of Incubation Conditions on the Thermal Stability of Eupergit C-Chymotrypsin and Penicillin G-acylase (PGA)[a]

Incubation conditions				
pH	T (°C)	Time (h)	Enzyme	Relative stability
7	20	0	Chromotrypsin	1
10	20	96	Chromotrypsin	6
10	4	96	Chromotrypsin	2
7	20	120	PGA	1
10	20	120	PGA	18
10	4	120	PGA	5

[a]Inactivations were carried out at pH 8.0 and 50°C (PGA) or 70°C and pH 7.0 (chymotrypsin).

5. If necessary, verify reference enzyme activity to evaluate the yield and the recovered activity of enzyme during the immobilization process.
6. Amounts and kind of EC-EP or Eupergit supports, ratios, V gel/V suspension used, and reaction times must be established for each enzyme. Avoid magnetic stirring to reduce abrasion of the support, especially for Eupergit supports.
7. If the enzyme activity is decreased because of enzyme inactivation it must be distinguished from a loss in enzyme activity of the supernatant resulting from immobilization process.
8. Multipunctual derivatives can be reached through an extended incubation period of enzyme suspension after the immobilization has concluded. Additional bonds may be formed by keeping the suspension at pH 10.0. The optimum multi-interaction time required for each enzyme must be established for every case. It is necessary to prepare enzyme derivatives with different multi-interaction times and check the thermal stability of each. The optimal time is the shortest period that provides optimal stability and enzyme recovery; this will be a compromise.

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5

Glutaraldehyde in Protein Immobilization

A Versatile Reagent

Lorena Betancor, Fernando López-Gallego, Noelia Alonso-Morales, Gisella Dellamora, Cesar Mateo, Roberto Fernandez-Lafuente, and Jose M. Guisan

Summary

The use of glutaraldehyde and supports containing primary amino groups is one of the most frequently used techniques for enzyme immobilization. However, glutaraldehyde is a very versatile reagent. Using low-ionic strength, the cationic nature of the surface permits the rapid ionic immobilization of the proteins. There are two different possibilities: (1) activate the support and immobilize the enzyme in a glutaraldehyde-activated support (in this case the immobilization is promoted by ionic exchange) or (2) adsorb the proteins on the aminated supports and treat the immobilized preparation with glutaraldehyde to cross-link both the enzyme and the support. Both alternatives have advantages and drawbacks that will be discussed on this chapter.

Key Words: Support–enzyme crosslinking; ionic adsorption; multipoint covalent attachment; enzyme stabilization; multipoint covalent attachment.

1. Introdution

Covalent immobilization of enzymes by means of glutaraldehyde chemistry is one of the most frequently used technologies for enzyme immobilization. There are several ways of using glutaraldehyde for this purpose, such as the immobilization of enzymes on supports previously activated with glutaraldehyde (*see* **Fig. 1**) ***(1–8)*** or the treatment with glutaraldehyde of proteins adsorbed on supports having primary amino groups (*see* **Fig. 2**) ***(9–10)***.

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Fig. 1. Protein immobilization on aminated supports pre-activated with glutaraldehyde.

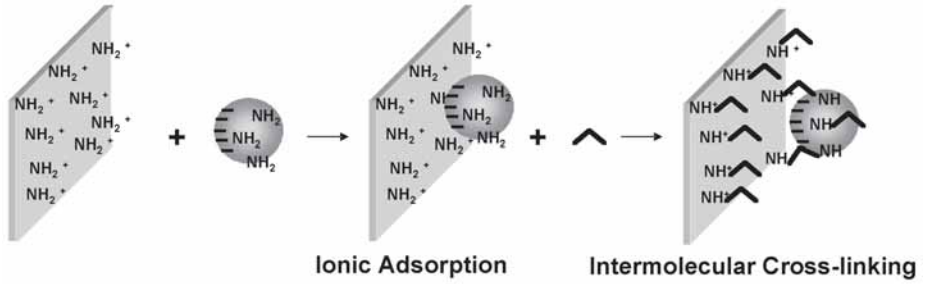

Fig. 2. Cross-linking with glutaraldehyde of proteins ionically adsorbed onto aminated cationic supports.

1.1. Protein Immobilization on Aminated Supports Preactivated With Glutaraldehyde

The immobilization of proteins on glutaraldehyde preactivated supports is quite simple and efficient, and in some instances even permits the improvement of enzyme stability by multipoint or multisubunit immobilization ***(7,8)***.

These supports are made by derivatization with glutaraldehyde of a matrix that originally must contain primary amino groups. This means that below each glutaraldehyde molecule, there are one or two amino groups (e.g., in the case of epoxy or aldehyde supports activated with ethylenediamine) ***(11)*** that can confer some ionic exchanger features to the support. Such supports can be considered heterofunctional matrixes in a manner similar to the recently described heterofunctional epoxy supports ***(12–15)***. And they take advantage of the possibility that an ionic exchange of the proteins occurs on the support before the covalent reaction is permitted (*see* **Fig. 1**) ***(16)***.

1.2. Cross-Linking With Glutaraldehyde of Proteins Ionically Adsorbed Onto Aminated Cationic Supports

Another strategy for immobilizing proteins using the glutaraldehyde chemistry is to treat proteins previously adsorbed on cationic supports containing primary amino groups with glutaraldehyde. The enzyme is first ionically adsorbed onto the ionic exchanger support and then treated with glutaraldehyde under mild condi-

tions. All the primary amino groups of the enzyme and support should be activated with one molecule of glutaraldehyde; the support–enzyme cross-linking may then occur. It has been shown that these glutaraldehyde groups (just one molecule per amino group) may promote intense cross-linking under a broad range of reaction conditions ***(17)***. It has also been shown that a similar result might be obtained using the glutaraldehyde groups of the activated enzyme and those from the support ***(18)***.

2. Materials

1. Aminated supports: MANAE agarose (Hispanagar S.A., Burgos, Spain), Aminopropil-CPG (Millipore billerica, MA), Aminoethyl agarose (Sigma-Aldrich St. Luis, MO), Sepabeads® EC-EA (Resindion SRL, Milan, Italy).
2. Immobilization buffer: 25 m*M* sodium potassium, pH 7.0.
3. 25% (v/v) Glutaraldehyde solution (Fluka, Switzerland).

3. Methods

3.1. Activation of Aminated Supports Activated With Glutaraldehyde

1. Prepare a 15% glutaraldehyde solution in 200 m*M* phosphate buffer and adjust the pH to 7.0 (*see* **Note 1**).
2. Suspend 10 mL of an aminated support in 20 mL of the glutaraldehyde solution prepared in **step 1** (*see* **Note 1**).
3. Gently stir the suspension for 15 h at 25°C (*see* **Note 2**). Filter and thoroughly wash the suspension with 5 vol 25 m*M* sodium phosphate buffer. Rinse thoroughly with distilled water. Store the gel at 4°C and use within 24 h.

3.2. Immobilization of Enzymes on Glutaraldehyde-Activated Supports

1. Suspend 10 g of the of glutaraldehyde support in 20 mL of enzymatic solution in immobilization buffer (*see* **Note 5**).
2. Gently stir at 25°C.
3. Withdraw aliquots from suspensions and supernatant and assay their catalytic activity. The frequency of sampling must be established for each immobilization.
4. Vacuum filter the derivative and wash thoroughly with distilled water.

3.3. Adsorption of Enzymes Onto Aminated Supports

1. Suspend 10 g of an aminated support in 20 mL of enzymatic solution prepared in immobilization buffer (*see* **Note 3**).
2. Gently stir at 25°C.
3. Withdraw aliquots from suspension and supernatant and assay their catalytic activity until total adsorption of the enzyme.
4. Wash the adsorbed enzyme thoroughly with distilled water and filter to dryness.

3.4. Cross-Linking of Adsorbed Derivatives With Glutaraldehyde

1. Prepare a 0.5% (v/v) glutaraldehyde solution (in 25 m*M* sodium phosphate buffer, pH 7.0.
2. Suspend 1 wet g of the adsorbed enzyme (*see* **Subheading 3.3., step 3**) in 4 mL of the glutaraldehyde solution.
3. Gently stir for 1 h at 25°C.

4. Filter and wash the modified immobilized enzyme thoroughly with 25 m*M* sodium phosphate buffer, pH 7.0, to remove the excess of glutaraldehyde. Filter to eliminate inter-particle water.
5. Keep for 20 h at 25°C and then store at 4°C (*see* **Note 4**).

3.5. Desorption of Noncovalently Immobilized Proteins on the Support

1. To check if the cross-linking has been successful, resuspend 0.5 g of derivative in 2.5 mL of 1 *M* sodium phosphate buffer, pH 7.0. Gently stir for 30 min at 20°C.
2. Assay the activity from the suspension and supernatant after the desorption process (*see* **Note 6**).

3.6. Immobilization–Stabilization of Glucose Oxidase Onto MANAE Agarose Preactivated With Glutaraldehyde

1. Prepare an enzymatic solution containing 13 U/mL 25 m*M* sodium phosphate buffer, pH 7.0.
2. Assay the catalytic activity of the enzymatic solution previously described. Add 10 g of MANAE agarose activated with glutaraldehyde to 20 mL of the glucose oxidase (GOX) solution and assay the enzyme activity of both the suspension and supernatant after 30 min at 25 C. If any activity remains in the supernatant stir the suspension for an additional 30 min under the same conditions. Repeat for the enzyme assays (*see* **Fig. 3**).
3. Filter and thoroughly wash the derivative with distilled water.
4. Evaluate the covalent immobilization as described previously (*see* **Subheading 3.5.**).
5. This preparation is more thermostable than the soluble enzyme (*see* **Fig. 4**).

3.7. Immobilization–Stabilization of D-Amino Acid Oxidase by Adsorption on Sepabeads™ EA-EC Plus Further Glutaraldehyde Treatment

1. Prepare a solution of D-amino acid occidase (DAAO) in 25 m*M* sodium phosphate, pH 7.0.
2. Assay the catalytic activity of this solution. Add 10 g of EC-EA to 20 mL of the previous DAAO solution. Measure the enzyme activity of both the suspension and supernatant after 30 min at 25°C.
3. Filter the derivative.
4. Add 1 g of derivative to 4 mL of 0.5% glutaraldehyde solution, pH 7.0. Gently stir for 1 h at 25°C.
5. Filter and wash suspension with the excess 25 m*M* sodium phosphate, pH 7.0. Incubate for 18 h at 25°C.
6. This derivative is more thermostable than the soluble enzyme (*see* **Fig. 5**).

3.8. Immobilization–Stabilization of Glutaryl Acylase by Adsorption on Sepabeads EA-EC Plus Further Glutaraldehyde Treatment

1. Prepare an enzymatic solution by mixing 5 mL of a commercial glutaryl acylase (GA) (Roche) with 20 mL of 25 m*M* potassium phosphate buffer, pH 7.0.
2. Assay the catalytic activity of the enzymatic solution previously described. Add 10 g of MANAE agarose to 20 mL of the GA solution and assay the enzyme activity of both the suspension and supernatant for 30 min at 25°C.

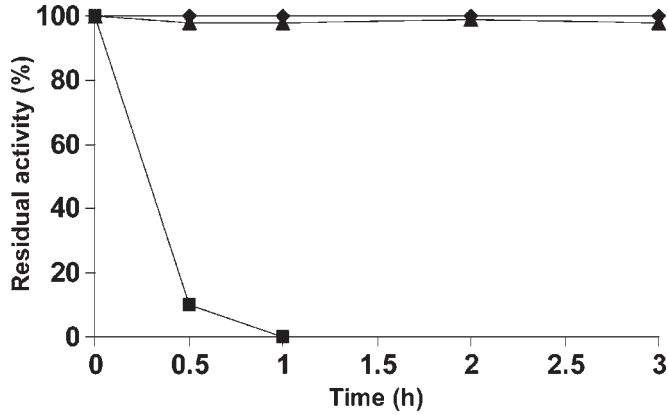

Fig. 3. Immobilization course of GOX on MANAE agarose activated with glutaraldehyde. Rhombus, control GOX solution; squares, supernatant, triangles, suspension. More details are described in **Subheading 3.6.**

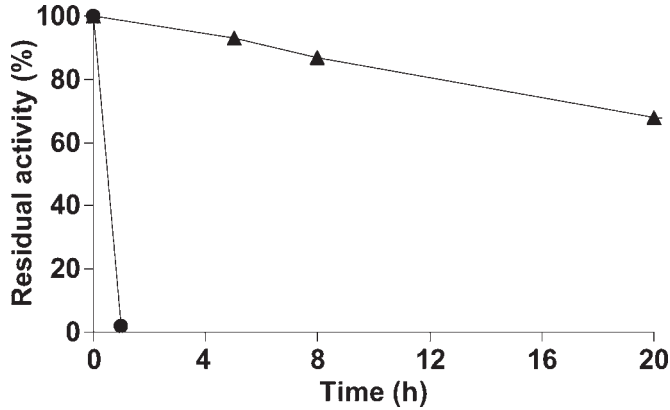

Fig. 4. Thermal stability of different GOX preparations. Triangle, immobilized on MANAE agarose activated with glutaraldehyde. Circle, soluble enzyme. Inactivation conditions were 56°C, pH 7.0, and 0.4 U/mL.

3. Filter the derivative. Add 1 g of derivative to 4 mL of 0.5% glutaraldehyde solution, pH 7.0. Leave for 1 h at 25°C.
4. Filter and wash the suspension with the excess 25 m*M* sodium phosphate, pH 7.0. Incubate for 18 h at 25°C.
5. Evaluate the covalent immobilization as described in **Subheading 3.5.**
6. This enzyme preparation is more thermostable than the soluble enzyme (*see* **Fig. 6**).

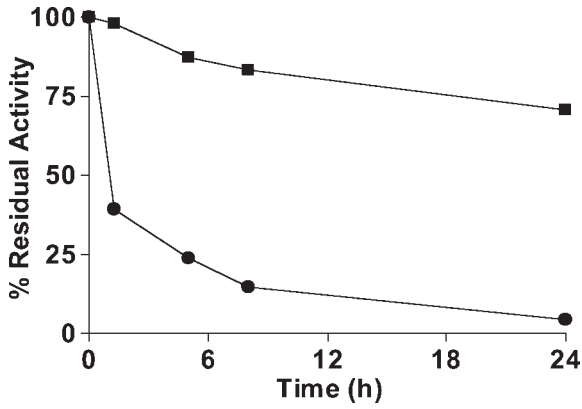

Fig. 5. Effect of cross-linking with glutaraldehyde on the thermal stability of DAAO. Circles, derivatives adsorbed onto Sepabeads EC-EA; squares, derivatives adsorbed onto Sepabeads EC-EA and then cross-linked with 0.5% glutaraldehyde solution. The inactivation course was carried out by incubating 0.8 U/mL DAAO in 10 m*M* potassium phosphate buffer, pH 7.0, at 50°C.

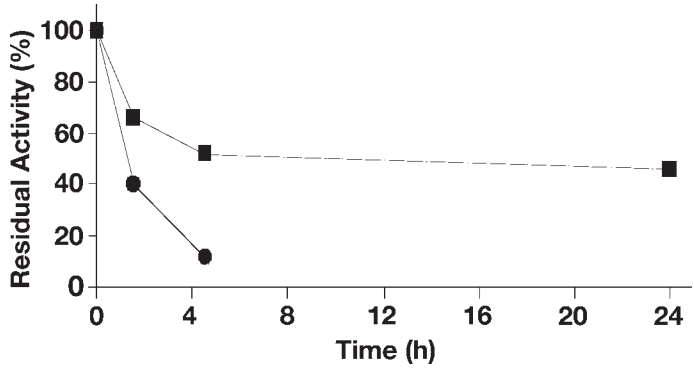

Fig. 6. Effect of cross-linking with glutaraldehyde on the thermal stability of the GA. Circles, derivatives adsorbed onto MANAE-agarose; squares, derivatives adsorbed onto MANAE agarose and then cross-linked with 0.5% glutaraldehyde solution. The inactivation course was carried out by incubating 10 U/mL of GA in 10 m*M* potassium phosphate buffer, pH 7.0, at 47°C.

4. Notes

1. The control of the pH is very important. If the pH is too high the glutaraldehyde will polymerize and the support will not react with the enzyme.
2. The suspension color is an indicator of the state of the glutaraldehyde. If the suspension is brownish in color the support cannot be used because glutaraldehyde reactivity is compromised.

3. The low-ionic strength of the immobilization buffer is necessary for the ionic adsorption between the protein and the support.
4. The additional incubation at 25°C is necessary to achieve a higher degree of cross-linking.
5. The low-ionic strength of the immobilization buffer permits the first ionic adsorption of the enzyme on the support. After this adsorption, the glutaraldehyde can covalently react with the primary amino groups of the protein, leaving the enzyme covalently attached to the support.
6. This experiment allows for the evaluation of the covalent attachment between the protein and the support.

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6

Practical Protocols for Lipase Immobilization Via Sol-Gel Techniques

Manfred T. Reetz

Summary

Lipases can be efficiently entrapped in the pores of hydrophobic silicates by a simple and cheap sol-gel process in which a mixture of an alkylsilane [$RSi(OCH_3)_3$ and $Si(OCH_3)_4$] is hydrolyzed under basic conditions in the presence of the enzyme. Additives such as isopropanol, polyvinyl alcohol, cyclodextrins, or surfactants enhance the efficiency of this type of lipase immobilization. The main area of application of these heterogeneous biocatalysts concerns esterification or transesterification in organic solvents, ionic liquids, or supercritical carbon dioxide. Rate enhancements (relative to the traditional use of lipase powders) of several orders of magnitude have been observed, in addition to higher thermal stability. The lipase immobilizates are particularly useful in the kinetic resolution of chiral esters, enantioselectivity often being higher than what is observed when using the commercial forms of these lipases (powder or classical immobilizates). Thus, because of the low price of sol-gel entrapment, the excellent performance of the lipase immobilizates, and the ready recyclability, this method is industrially viable.

Key Words: Lipases; sol-gel immobilization; esterification; transesterification; thermal stability; kinetic resolution; enantioselectivity; recyclability.

1. Introduction

A wide variety of enzymes are available to the practicing organic chemist for many different transformations ***(1,2)***. A milestone in the application of enzymes as catalysts for synthetic organic chemistry was the discovery that numerous enzymes can be used in nonaqueous media, allowing transformations of interest to organic chemists to be performed that were not possible in the natural aqueous environment ***(3)***. An example of significant synthetic importance is the use of lipases (EC 3.1.1.3) as catalysts in organic solvents. These enzymes are the most used biocatalysts in synthetic organic chemistry, catalyzing the hydrolysis of carboxy-

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lic acid esters in aqueous medium, or the reverse reaction (esterification), as well as transesterification in organic solvents ***(1–4)***. When working in organic media, nucleophiles other than alcohols can be used (e.g., amines or H_2O_2 affording amides and acid peroxides). Numerous examples involving enantioselectivity in the production of chiral alcohols, amines, and carboxylic acids have been reported. Lipases are structurally characterized by a so-called lid. When hydrophobic substrates interact with certain hydrophobic regions of a lipase, the lid opens and thus exposes the active site (serine) in a process called interfacial activation ***(4–8)***.

In the case of reactions in organic solvents (which are most often used), commercially available lipase powders are often employed. In spite of the obvious advantages in such simple protocols, several drawbacks need to be considered, primarily the considerably reduced lipase activities relative to those observed in aqueous medium and the extreme difficulty in recycling the enzyme. Thus, for real (industrial) applications of lipases some form of immobilization that not only allows for efficient separation and re-use of the enzyme but also leads to a significant enhancement of catalyst activity is necessary. Several approaches have been described in this book (*see* Chapters 2 and 13). The present chapter focuses on the entrapment of lipases in hydrophobic sol-gel silicates.

Sol-gel encapsulation has proven to be a particularly easy and effective way to immobilize enzymes ***(9)***. Following isolated reports describing specific examples ***(10,11)***, it was the seminal work of Avnir and coworkers that led to the generalization of this technique ***(9,12–14)***. Sol-gel techniques involve the acid- or base-catalyzed hydrolysis of tetraalkoxysilanes [$Si(OR)_4$] ***(15,16)***. Mechanistically, the silane-precursor undergoes hydrolysis and cross-linking condensation with formation of a SiO_2 matrix in which the enzyme is encapsulated. This type of encapsulation works well for a number of enzymes ***(9,12–14)***. However, in the case of lipases, materials were obtained that showed disappointingly low enzyme activities, as measured by the rate of the model reaction involving the esterification of lauric acid (**Fig. 1**, **1**), by *n*-octanol (**2**) in isooctane as solvent ***(17)***. Only a 5 to 10% activity relative to the traditional use of the respective lipase powder was observed, equivalent to relative rates of 0.05 to 0.1.

It was speculated that the microenvironment in SiO_2 may be too polar, and therefore mixtures of $Si(OCH_3)_4$ (**Fig. 2, 5**) and alkylsilanes of the type $RSi(OCH_3)_3$ (**4**) or polydimethylsiloxane (PDMS) having nonhydrolyzable lipophilic alkyl groups (R) were tested ***(17)***. This strategy was developed because the silicon oxide matrix is now hydrophobic, which can facilitate or simulate a type of interfacial activation of the entrapped lipase. Basic catalysts such as NaF were used for the sol-gel process, because such conditions lead to large pores in the silicate matrix. Indeed, dramatically improved relative lipase activities typically amounting to 200 to 800% were observed in the model reaction, which corresponds to an enhancement of relative enzyme activity by a factor ranging from two to eight with respect to the traditional use of the corresponding lipase powder (lyophilizate) ***(17,18)***. Relative activity is defined as [v(immobilized lipase)/v(commercial lipase)], where v is the initial rate of the reaction in each case. A pronounced increase in thermal stability was also observed. In most cases the optimal ratio of $RSi(OCH_3)_3$ to $Si(OCH_3)_4$ turned out to be about 5:1, although it was not possible to present an experimental protocol that was completely general.

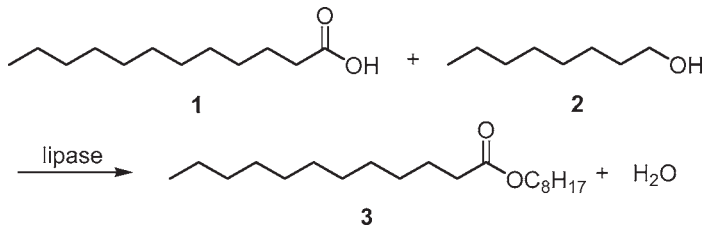

Fig. 1. Model esterification of lauric acid **(1)** by *n*-octanol **(2)** catalyzed by a lipase.

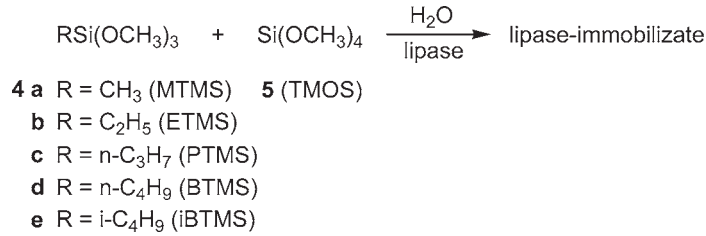

Fig. 2. Encapsulation of a lipase in hydrophobic sol-gel materials.

Usually methyltrimethoxysilane $CH_3Si(OCH_3)_3$ (MTMS) was used as the precursor and polyvinyl alcohol (PVA) as an additive, the latter possibly acting as a stabilizer of the lipase ***(17,18)***.

An important extension of this method pertains to the use of additional porous solid supports during the sol-gel process ***(19)***. This type of "double immobilization" involves binding of the lipase-containing gels in the pores of the solid support (e.g., silicates of the type SIRAN® or Celite®) as gelation occurs (*see* **Fig. 3**). Such a process results in higher mechanical stability and enhanced enzyme activity (up to a factor of 88).

It should be noted that sol-gel *encapsulation* is crucial in both variants because conventional *adsorption* on hydrophobic silicates or on SIRAN alone affords poor catalysts. The structural and morphological properties of the lipase immobilizates were characterized by scanning electron microscopy (SEM), solid state ^{29}Si and ^{13}C NMR spectroscopy, and in studies concerning specific surface area and pore volume ***(20)***. Moreover, kinetic studies clearly point to an "alkyl effect" (i.e., enhancement of lipase-activity upon using $RSi(OCH_3)_3$ in the series methyl < ethyl < *n*-propyl < *n*-butyl) ***(20)***. Enhanced hydrophobicity in the silicon oxide matrix correlates with increased enzyme activity. Higher thermal stability and activity appear to result from multipoint interactions through hydrogen bonding as well as ionic and hydrophobic interactions (van der Waals), which can be schematized as shown in **Fig. 4**. Hydrophobic interactions can result in a type of interfacial activation. The lipase may be conformationally arrested in the matrix in a "lid-opened" and therefore active form. The early work on lipase entrapment in hydrophobic sol-gel materials has been reviewed ***(21)***.

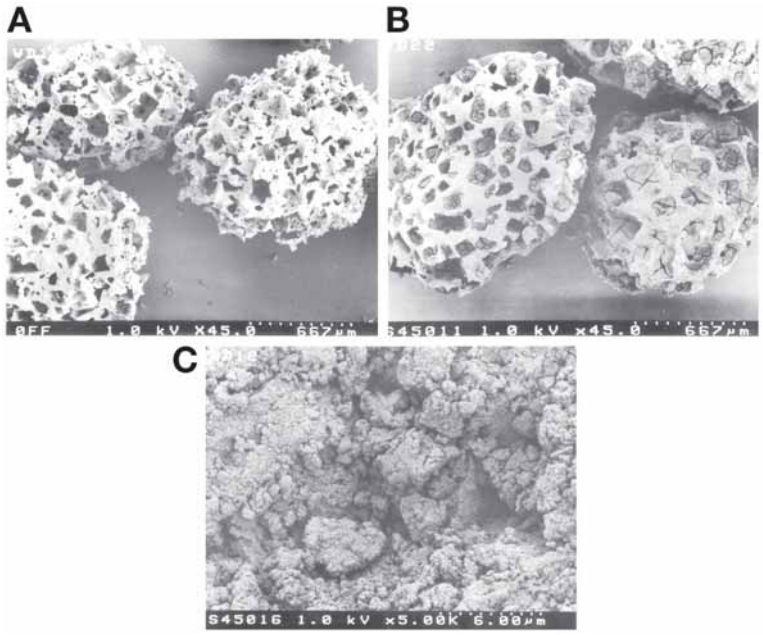

Fig. 3. (**A**) SEM image of untreated SIRAN showing hollow pores in which the lipase-containing sol-gel material can be bound. (**B**) SEM image of a lipase SP 523 containing TMOS/PDMS (4:1) gel in the pores of SIRAN. (**C**) Approximate 100-fold magnification of the SEM image of (**B**) ***(19)***.

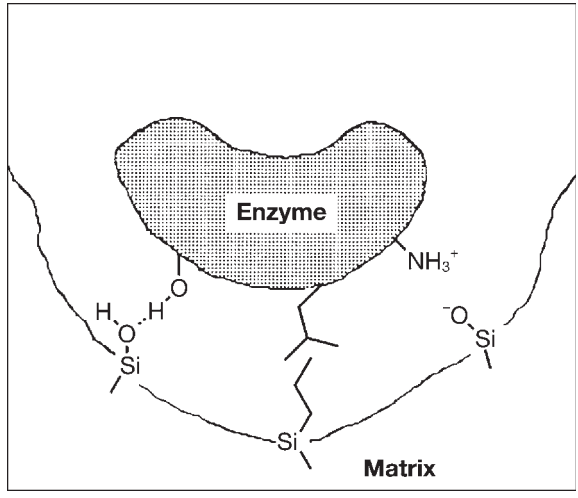

Fig. 4. Schematic view of noncovalent interactions between the gel matrix and the lipase ***(21)***.

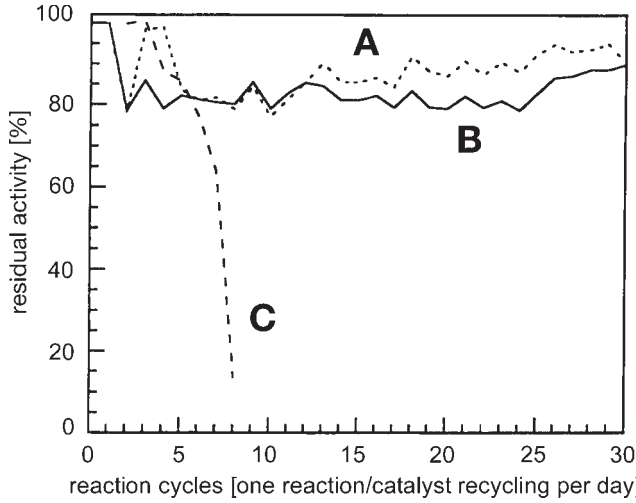

Fig. 5. Activity of immobilized lipase PS (Amano) after repeated use in esterification reactions involving **1** + **2** → **3**. (**A**) MTMS/PDMS (6:1) gel with entrapped lipase PS. (**B**) MTMS gel with entrapped lipase PS. (**C**) MTMS gel with adsorbed lipase PS ***(18,21)***.

Successful recycling of sol-gel lipase immobilizates derived from eight different lipases was demonstrated ***(18,21)***. For example, the entrapped lipase from *Pseudomonas cepacia* in MTMS or MTMS/PDMS gels were repeatedly used in batch esterification involving the model reaction of **1** and **2**. After shaking the reaction mixture at 30°C for 23 h, the lipase-containing gels were recovered by filtration or centrifugation, washed with isooctane and pentane, and reused. In addition, the gels were washed with acetone after every fifth reaction. After a slight loss of enhanced activity, probably resulting from a loss of surface adsorbed lipase, enzyme activity remained constant for at least 30 reactions at about 85% of the original high value. Under the same conditions a control experiment in which the lipase was physically *adsorbed* to the hydrophobic gel showed that the material loses its activity completely after only a few reactions (*see* **Fig. 5**) ***(18,21)***. These experiments illustrate an important point, namely that immobilization by adsorption is certainly viable in this system (and in other types of immobilizates), but only for a very limited number of cycles involving reaction/recyclization. This phenomenon is not always reported in the literature.

Although the sol-gel lipase immobilizates were designed for application in nonaqueous media, they can also be used as heterogeneous catalysts for ester hydrolysis in aqueous medium ***(22)***. Another development concerns the possibility of magnetic separation as a means to recycle the lipase catalysts, specifically by incorporating magnetite (iron oxide) during the sol-gel process ***(23)***. Moreover, the concept of lipase entrapment in hydrophobic silicates has been extended to aerogels ***(24,25)***.

Significant progress was made possible by the development of second-generation sol-gel lipase immobilizates ***(26)***. They opened the way to heterogeneous cata-

lysts which are more active while at the same time retaining enhanced thermal and mechanical stability and the possibility to recycle efficiently. It involves the optimal choice of the alkyl group in the sol-gel precursor $RSi(OCH_3)_3$ (**Fig. 2**, **4**) as well as the use of additives such as 18-crown-6, Tween-80, cyclodextrins, isopropanol, and/or KCl. Such additives had been previously used to activate lipases in the absence of sol-gels ***(27–37)***. The most active lipase gels are based on *n*-butyl- or isobutylsilane precursors (**Figs. 2**, **4d,e**) and contain either 18-crown-6 or Tween-80 as additives ***(26)***. Nine different lipases (PfL, BcL, MmL, AnL, CrL (type VII), CrL(L-3), TIL, PpL and PrL as described in **Subheading 3.**) were subjected to sol-gel immobilization of the second generation, and all of them displayed improved catalytic performance ***(26)***. In some cases, "double" immobilization (i.e., performing the sol-gel lipase entrapment not only in the presence of an appropriate additive, but also in the presence of a solid support such as SIRAN [*see* **Fig. 3**]) or Celite led to additional enhancement of activity as well as thermal and mechanical stability. Although this actually increases the amount of solid support (Celite plus attached sol-gel silicate) relative to the amount of lipase, less total heterogeneous catalyst needs to be employed weight-wise. This is because the enzyme activity in the model reaction is dramatically higher relative to the use of lipase powders. For example, in the case of the lipase TIL, the factor is 1391 (relative activity), corresponding to an activity of 1237 µmol/min·g gel and a specific activity of 8899 mmol/min·g protein. Sol-gel encapsulation of lipases also enhances enantioselectivity in most reactions tested, as in the kinetic resolution of *rac*-6 (**Table 1** and **Fig. 6**) ***(26)***.

It has been noted that in many protocols concerning biocatalysis (including lipases) in nonaqueous medium more enzyme than substrate is used weight-wise ***(38)***. When employing second-generation sol-gel lipase immobilizates as heterogeneous catalysts, this is not the case ***(26)***. In a typical preparative scale reaction, only about 250 mg of a lipase-containing silica gel are needed for 10 g of substrate, as demonstrated in the kinetic resolution of *rac*-9 (**Fig. 7**). This is all the more significant when recalling that most of the heterogeneous catalyst is in fact weight-wise the silicate matrix.

In summary, the second-generation sol-gel lipase immobilizates ***(26)*** are considerably more active and enantioselective than the original first-generation materials ***(17,18,21,39–43)***. They constitute industrially viable heterogeneous biocatalysts.

2. Materials

2.1. Lipase-Catalyzed Reactions

1. Lauric acid (Fluka, Switzerland).
2. *n*-Octanol (Fluka).
3. *rac*-2-Octanol (Aldrich, Germany).
4. Vinyl acetate (Acros, UK).
5. *rac*-2-Naphthyl-2-ethanol (Aldrich).
6. Isooctane (Fluka).
7. Toluene (Overlack, Germany).

Table 1
Kinetic Resolution of Alcohol[a] Using Traditional Lipase-Powders and Sol-Gel Encapsulated Lipases as Catalysts, Vinyl Acetate[b] as the Acylating Agent, and Isooctane as Solvent *(26)*

Entry	Lipase	Additives used in sol-gel process	Activity (μmol/min·g gel)	Specific activity (μmol/min·g protein)	Relative activity	Selectivity factor (E)
1	CaLB (powder)	None	100	188	–	70.1
2	CaLB (sol-gel)	None	529	790	4.2	>100
3	CaLB (sol-gel)	18-crown-6	425	966	5.1	>100
4	BcL (powder)	None	6	51	–	4.4
5	BcL (sol-gel)	None	14	288	5.6	4.1
6	BcL (sol-gel)	18-crown-6	45	964	18.8	4.0
7	BcL (sol-gel)	Tween-80®	28	937	18.3	4.5
8	PfL (powder)	None	6	34	–	2.2
9	PfL (sol-gel)	None	28	108	3.2	3.3
10	PfL (sol-gel)	18-crown-6	40	625	18.4	6.0

[a]**Fig. 6**, **6**.
[b]**Fig. 6**, **7**.

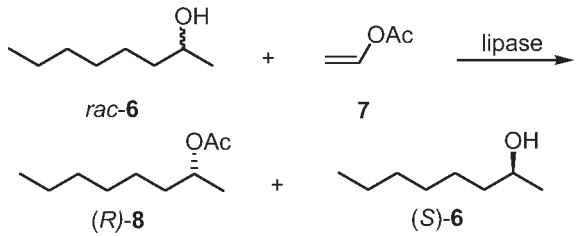

Fig. 6. Kinetic resolution of *rac*-**6** catalyzed by lipases.

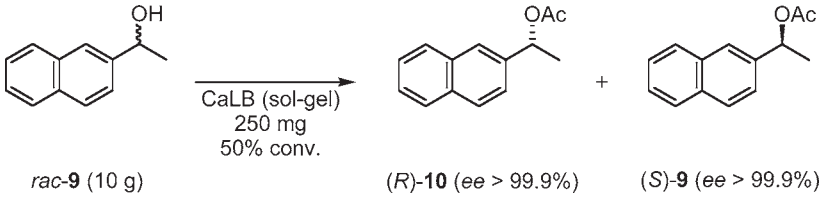

Fig. 7. Kinetic resolution of *rac*-**9** catalyzed by lipases.

2.2. Sol-Gel Process

1. *n*-Butyltrimethoxysilane (ABCR, Germany).
2. Isobutyltrimethoxysilane (Lancaster, UK).
3. Tween-80 [poly-oxyethylene (20) sorbitan monooleate] (Fluka).
4. Sodium fluoride (Fluka).
5. Celite 577 (Fluka).
6. Methyl-β-cyclodextrin (Aldrich).
7. Tetramethoxysilane (Fluka).
8. Polyvinyl alcohol (PVA; molecular weight [mol wt] = 15000) (Merck, Germany).

2.3. Lipases

The lipases from *Candida antarctica* (CaLB; Chirazyme L-2®), *C. rugosa* (CrL; Chirazyme L-3®), and *Mucor miehei* (MmL; ChirazymeL-9®) are accessible from Roche; *Aspergillus niger* (AnL; Amano AS), *Burkholderia cepacia* (BcL; Amano PS), and *Pseudomonas fluorescens* (PfL; Amano AK "20") from Amano Pharmaceutical Co.; *C. rugosa* type VII (CrL type VII) from Sigma; *Penicillium roqueforti* (PrL) from Fluka; *Thermomyces lanuginosa* (TIL; Novozym SP) from Novo Nordisk.

3. Methods

3.1. Sol-Gel Entrapment of Lipases General Procedure

1. A commercial lipase powder (lyophilizate), such as AnL (150 mg), BcL (150 mg), CaLB (125 mg), CrL (150 mg), CrL type VII (60 mg), MmL (150 mg), PfL (150 mg), PpL (150 mg), PrL (150 mg), or TIL (70 mg) is placed in a 50-mL Falcon tube (Corning) with 390 µL, 0.1 *M* Trid/HCl-buffer, pH 7.5, and the mixture is vigorously shaken with a vortex mixer. In the case of an additive or an additional porous solid support, these materials are included as needed ***(26)***. In a typical procedure, 0.5 mmol of 18-crown-6 as an additive is added. In the case of "double immobilization," the additive is used as above in addition to 50 mg of Celite.
2. Then 100 µL of aqueous 4% (v/v) PVA, 50 µL of 1 *M* aqueous sodium fluoride, and 100 µL isopropanol are added and the mixture is homogenized using a vortex mixer.
3. Then the alkylsilane (**Fig. 2**, **4d** or **4e**) (2.5 mmol) and TMOS (**Fig. 2**, **5**) (0.5 mmol; 74 µL; 76 mg) are added. The mixture is agitated once more for 10 to 15 s. Gelation is usually observed within seconds or minutes while the reaction vessel is gently shaken.
4. Following drying overnight in the opened Falcon tube, 10 to 15 mL isopropanol is added in the order to facilitate removal of the white solid material (filtration).
5. The gel is washed with 10 mL of distilled water, 10 mL isopropanol, 10 mL and *n*-pentane. During this process a spatula is used to crush the gel.
6. The lipase immobilizate is placed in an open 2-mL plastic vessel and dried at room temperature ***(26)***.

3.2. Determination of Protein Content

In order to determine the protein content of the commercial lipases as well as the degree of loading, the *BCA Protein Assay Kit* (Sigma) can be used. Accordingly, solutions of the commercial lipase or the wash-solutions following the sol-gel process are incubated for 30 min and measured at 37°C using a UV/Vis-spectrometer at 562 nm according to the *Technical Bulletin* (Sigma). Distilled water is used as a reference and bovine serum abumin (BSA) as standards. The degree of immobilization ranges between 0.3 and 0.9 ***(26)***.

3.3. Determination of Lipase Activity

1. Depending on the activity, between 1 and 50 mg of the sol-gel lipase-immobilizate is placed in a Falcon tube together with solutions of lauric acid (100 mg; 0.5 mmol) solution and *n*-octanol (158 µL; 130 mg; 1.0 mmol) in nondried (H_2O-saturated) isooctane.
2. The mixture is then shaken at 180 rounds per minute (rpm) at 30°C. At defined intervals (usually after 15, 30, 45, and 60 min) 300 µL samples are taken and centrifuged (13,000 rpm) before the gas chromatographic determination of lauric acid and lauric acid *n*-octyl ester is carried out using 150 µL of the supernatant.

3. By applying a linear regression of the measured values the initial reaction rate in µmol/min and therefore the activity relative to 1 g of immobilizate can be determined. By considering the degree of loading, the specific activity is calculated.
4. The relative activity is determined by dividing the specific activity of the immobilizate by the specific activity of the commercial lipase powder ***(26)***.

3.4. Typical Kinetic Resolution on a Preparative Scale

1. Similar to the above protocol a mixture of *rac*-1-(2-naphthyl)-ethanol (10 g; 58 mmol), vinyl acetate (8.1 mL; 7.5 g; 87 mmol), and 250 mg of a sol-gel CaLB-immobilizate (prepared in the presence of 18-crown-6 as additive) in 300 mL toluene is shaken at 35°C for 48 h.
2. Following filtration gas chromotography analysis shows a conversion of 50.0%, the enantio meric excess of no-reacted (*S*)-1-(2-naphthyl)ethanol and product ((*R*)-acetate) each being >99.9%.
3. The immobilizate is removed by filtration, washed with toluene and pentane, and can be reused, showing no significant loss in activity or enantioselectivity ***(26)***.

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7

Encapsulation of Enzymes Using Polymers and Sol-Gel Techniques

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Summary

This chapter describes two enzyme immobilization methods based on the biomolecule encapsulation into polymer matrices: the sol-gel technology and the entrapment into the polymer poly(vinyl alcohol) with styrylpyridinium groups (PVA-SbQ). The sol-gel technology is based on the formation of silica matrices of metal or semi-metal oxides through the aqueous processing of hydrolytically labile precursors. The encapsulation into PVA-SbQ involves the photo-cross-linking of the styrylpyridinium groups in order to create the polymer matrix. These networks are chemically stable and do not restrict the enzyme activity. Both bioencapsulation strategies provide simple, easy, and low-cost methods for enzyme immobilization. They are versatile, as matrixes can be tailor-designed and used to entrap a large number of biomolecules. They present numerous applications, including the development of biooptical devices, biosensors and biocatalysts.

Key Words: Entrapment; sol-gel technology; tetramethoxysilane; TMOS; methyltrimethoxysilane; MTMOS; poly(vinyl alcohol) bearing styrylpyridinium groups; PVA-SbQ; photo-cross-linking; polymer matrix; screen-printed graphite electrode.

1. Introduction

Biosensors, bioarrays, lab-on-a-chips, biocatalysts, and industrial bioprocesses are some examples of technologies that have as a common key point the immobilization of biological materials such as enzymes, engineered proteins, catalytic antibodies, nucleic acids, and whole cells. All of these biotechnologies require a successful bio-immobilization in terms of resistance to leaking or desorption, long-term stability, stability under adverse experimental conditions, retention of the biomolecule functionality, or activity, accessibility to analytes, fast response times, and sometimes high immobilization density and oriented immobilization.

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Among the different methods for enzyme immobilization, encapsulation is of particular interest because of the simplicity of preparation and the biomolecule freedom. The fabrication process is straightforward and reproducible and does not require sophisticated equipment. The encapsulation process is based on the entrapment of the biomolecule in a polymer matrix and there is no covalent association between the network and the biomolecule. This entrapment restricts rotation and unfolding movements but allows substrate recognition and binding as well as catalysis ***(1–4)***. Other advantages are the permeability of the matrices, which allows the transport of low-molecular-weight (MW) compounds without leaking of the entrapped biomolecules; the tuneable material porosity, which allows the accommodation of biomolecules of different size; the possibility of chemically modifying the matrix, introducing, for example, electrochemical moieties; the optical properties, which open the possibility to measure absorbance of fluorescence signals; the resistance to chemical, thermal, and biological degradation; and the negligible swelling effects.

In this chapter, two different encapsulation methods are described: the sol-gel technology and the bioencapsulation in a matrix formed by photo-cross-linkable poly(vinyl alcohol)-bearing styrylpyridinium groups (PVA-SbQ). The sol-gel process (*see* **Fig. 1**) is based on the ability to form metal-oxide, silica, and organosiloxane matrices of defined porosity by the reaction of organic precursors at room temperature ***(5)***. In a first reaction, one or two metal alkoxide precursors (usually, tetramethoxysilane [TMOS; $Si(OCH_3)_4$] and/or methyltrimethoxysilane [MTMOS; $H_3Si(OCH_3)_3$] are hydrolyzed in the presence of water at acid pH, resulting in the formation of silanol (Si-OH) groups. In the second step, the condensation reaction between silanol moieties at basic pH results in the formation of siloxane (Si-O-Si) polymers, creating a matrix in which the biomolecules are entrapped. In other words, the hydrolysis of precursors results in the sol formation and subsequent gelation occurs by their condensation and polycondensation. Finally, the sol-gel is dried, forming a xerogel. In the PVA-SbQ encapsulation (*see* **Fig. 2**), the photo-cross-linking of the styrylpyridinium groups of the PVA-SbQ creates a network in which the biomolecule is entrapped

The encapsulation techniques are not restrictive in terms of the type of enzyme that can be immobilized. Sol-gel encapsulation started with the entrapment of glucose oxidase as a model enzyme, using different sol-gel-derived materials ***(6–9)***. The versatility of this enzyme has led to the use of different strategies for its detection based on electrochemical and optical measurements. Horseradish peroxidase has also been immobilized using sol-gel processes, leading to biosensors for different analytes, such as hydrogen peroxide ***(10)*** and cyanide ***(11)***, with high sensitivity, high stability, and high reproducibility. With the purpose to determine phenols, tyrosinase has also been entrapped by sol-gel techniques. This enzyme retained 73% of its original activity after intermittent use for 3 wk when stored in a dry state at 4°C ***(12)***.

The PVA-SbQ encapsulation technique has also provided the entrapment of different enzymes. Aldehyde dehydrogenase was immobilized by PVA-SbQ entrapment on the surface of disposable screen-printed graphite electrodes with the purpose of developing a biosensor for the detection of metam-sodium, a slightly

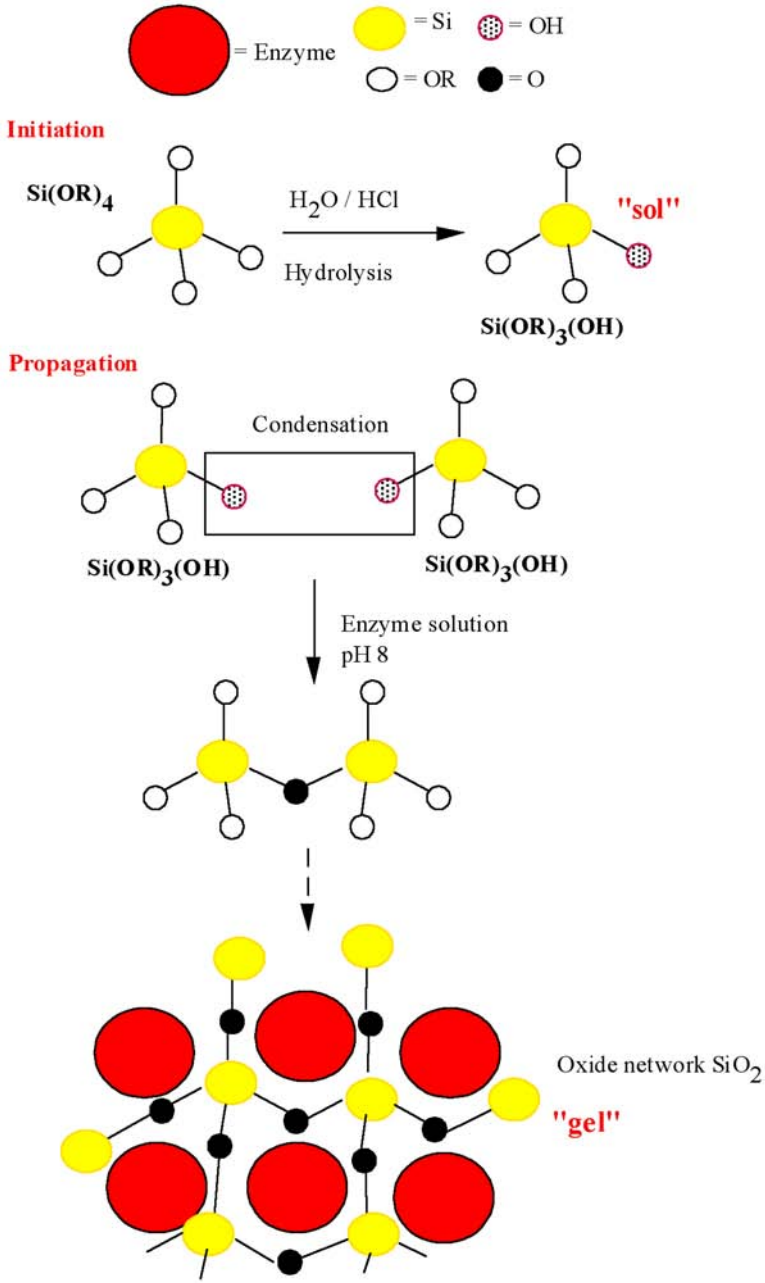

Fig. 1. The sol-gel entrapment of enzymes. (From **ref. *26***.)

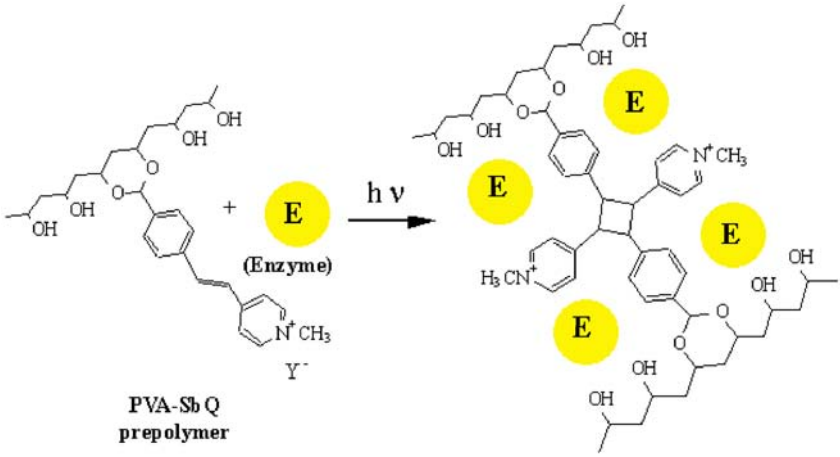

Fig. 2. The PVA-SbQ entrapment of enzymes. (From **ref. *13***.)

toxic soil fumigant, and its toxic metabolite methyl isothiocyanate (MITC) ***(13)***. The enzyme activity was inhibited by the presence of these dithiocarbamate fungicides, although the biosensor showed a relatively low sensitivity (the limit of detection for MITC being 100 ppb), probably as a result of the high amount of immobilized enzyme. This mono-enzymatic biosensor was stable during 23 successive injections, indicating that the retention of the enzyme was successful. Bi-enzymatic biosensors incorporating aldehyde dehydrogenase and diaphorase were also developed for the detection of other dithiocarbamante fungicides ***(14)***. The lowest limits of detection were 0.05 ppm and 0.25 ppm for maneb and zineb, respectively. In this case, a 60% desorption of the diaphorase enzyme was observed when using a PVA-SbQ polymer with a low degree of polymerization (1700), which reduced the operational stability by 60% after 10 assays. This operational stability was improved by the incorporation of a cellophane membrane on top of the electrode surface ***(15)***. In this case, the biosensor was able to detect as low as 1.48 ppb of maneb. The same bi-enzymatic strategy was used for the detection of acetaldehyde in wines, beers, and ciders, achieving limits of detection comparables to those obtained with the standard spectrophotometric method ***(16)***. Through the co-entrapment of aldehyde dehydrogenase, nicotinamide-adenine dinucleotide (NADH) oxidase and high-MW NADs (NAD-dextran and NAD-polyethylene glycol [PEG]), a reagentless acetaldehyde sensor was developed ***(17)***. The high-MW NADs were used to avoid the leaking that would be observed if the low-MW NAD^+ cofactor had been used in the encapsulation. The sensor reported limits of detection of acetaldehyde in alcoholic beverages comparable to those obtained by spectrophotometry. Moreover, the sensor did not show any decrease in the operational stability after 80 successive measurements. D-Lactate dehydrogenase was another of the dehydrogenases encapsulated using PVA-SbQ. The developed biosensor was used for the detection of D-lactate in French and Romanian wines ***(18)***. Finally, the

entrapment of acetyl cholinesterase allowed the detection of paraoxon (19.1 n*M*) and chlorpyrifos ethyl oxon (1.24 n*M*) in the presence of 5% acetonitrile ***(19)***.

It is not just different enzymes that can be immobilized using the encapsulation techniques but other biomolecules as well. Antibodies, regulatory proteins, membrane-bound proteins, and whole cells have been entrapped using this generic and versatile technology ***(20–24)***. However, each biomolecule requires specific component ratios, and optimization of the experimental parameters always has to be performed.

Despite all the advantages and the promising results achieved, it is necessary to keep in mind that bioencapsulation is still in its early phases. More biocompatible precursors and protocols are needed, shrinkage and pore-collapse effects have to be reduced, and the porosity and mechanical stability of sol-gels has to be improved if better catalytic efficiencies and response profiles are to be achieved ***(25)***. In the PVA-SbQ encapsulation, the leaking of the biomolecule from the polymeric network has to be completely eliminated and the reproducibility has to be guaranteed ***(14,26)***. In any case, despite the nonoptimized biocompatibility, porosity, mechanical robustness, and long-term stability of the entrapped enzymes, the encapsulation techniques are attractive and reliable tools for bio-immobilization in many practical applications such as bio-optical devices, biotransducers, biosensors, biocatalysts, bioaffinity chromatography, bioelectronics, and bioprocesses.

2. Materials

2.1. Sol-Gel Encapsulation

1. Tetramethoxysilane (TMOS; Sigma, St. Louis, MO). **Caution**: corrosive.
2. Methyltrimethoxysilane (MTMOS; Sigma). **Caution**: highly flammable.
3. Milli-Q water.
4. 1 m*M* HCl. **Caution**: corrosive.
5. Polyethylene glycol (PEG) with average mol wt: 600 (PEG_{600}; Sigma).
6. Enzyme to be immobilized.
7. Automatic pipets (and automatic pipets special for viscous solutions).
8. Eppendorf tubes.
9. Vortex mixer.
10. Screen-printed graphite electrodes (BIOMEM Group, Université de Perpignan, France).
11. Refrigerator or cold chamber.

2.2. PVA-SbQ Encapsulation

1. Enzyme to be immobilized.
2. Milli-Q water.
3. Photo-cross-linkable poly(vinyl alcohol)-bearing styrylpyridinium groups (PVA-SbQ) with degree of polymerization: 1700, degree of saponification: 88, SbQ content: 1.1 mol %, solid content: 11 mol %, pH 7.0 (Toyo Gosei Kogyo Co., Chiba, Japan).
4. Automatic pipets (and automatic pipets special for viscous solutions).
5. Eppendorf tubes.

6 Vortex mixer.
7. Benchtop centrifuge.
8. Screen-printed graphite electrodes (BIOMEM Group, Université de Perpignan), Maxisorp microtiter plates (Nunc, Roskilde, Denmark) or Ultrabind modified polyethersulfone affinity membranes with 0.45 µm pore size (Pall Gelman Sciences Inc., New York).
9. Neon light (e.g., 15 W lamps × 2).
10. Refrigerator or cold chamber.

3. Methods

3.1. Sol-Gel Encapsulation

1. Mix the reactant solutions TMOS, MTMOS, H_2O, HCl (1 m*M*) and PEG_{600} in the convenient ratio (*see* **Note 1**) in a 1.5-mL Eppendorf tube using automatic pipets (use an automatic pipet special for viscous solutions for PEG_{600}).
2. Cap the Eppendorf tube and sonicate for 15 min to homogenize the mixture.
3. Store the Eppendorf tube at 4°C overnight to allow hydrolysis of the precursors (*see* **Note 2**).
4. Dissolve the enzyme in a basic buffer using Milli-Q water (*see* **Note 3**).
5. Mix the sol solution with the enzymatic solution in a 50:50 ratio in a 1.5-mL Eppendorf tube and using automatic pipets to start the condensation (*see* **Notes 4–6**).
6. Cap the Eppendorf tube and homogenize the mixture using a vortex mixer (*see* **Note 6**).
7. Spread the mixture on the support surface using an automatic pipet (*see* **Notes 6–8**).
8. Dry the support surface for at least 36 h at 4°C (*see* **Note 9**).
9. Rinse the support surface with water prior utilization (*see* **Note 10**). While rinsing, carefully look to see if any desorption of the sol-gel from the surface support occurred.

3.2. PVA-SbQ Encapsulation

1. Dissolve the enzyme in the convenient buffer using Milli-Q water.
2. Mix the enzymatic solution with PVA-SbQ in a 50:50 ratio in a 1.5-mL Eppendorf tube and using automatic pipets (use an automatic pipette special for viscous solutions for PVA-SbQ) (*see* **Notes 11** and **12**).
3. Cap the Eppendorf tube and homogenise the mixture using a vortex mixer (*see* **Note 13**).
4. Spread the mixture on the support surface using an automatic pipet (*see* **Notes 7** and **14**).
5. Expose the support surface to neon light for 3 h at 4°C to allow entrapment of enzymes by polymerization (*see* **Note 15**).
6. Dry the support surface for at least 36 h at 4°C (*see* **Note 9**).
7. Rinse the support surface with water prior utilization (*see* **Note 10**). While rinsing, carefully look to see if any desorption of the PVA-SbQ from the surface support occurred.

Table 1
Precursor Mixture Compositions in μL (Final Volume: 108 mL).

TMOS	10	10	10	2	2	2	5	5	5
MTMOS	10	10	10	20	20	20	10	10	10
H_2O	44	88	132	44	88	132	44	88	132
HCl	40	80	120	40	80	120	40	80	120
PEG_{600}	4	4	4	4	4	4	4	4	4

4. Notes

1. Suggested ratios are shown in **Table 1**. However, other ratios, as well as other precursors, can be used. It should be taken into account that the higher the TMOS proportion, the faster the subsequent polymerization process.
2. Hydrolysis time depends on each case and should be optimized.
3. At basic pH, the condensation is favored. The appropriate basic pH will depend on the enzyme performance (not too high to inactivate it). The buffer composition is not particularly important. However, $(NH_4)_2SO_4$ should not be used to avoid precipitation.
4. The 50:50 ratio is suggested. However, other ratios can be used.
5. Controls are usually performed without enzyme but using the basic buffer.
6. Sometimes the condensation occurs at faster rates. As a result the sol-gel starts to form before its deposition on the support surface. It is then convenient to carry out this step at 4°C (cold chamber).
7. The volume of mixture deposited on the support surface depends on the particular interest, but it has never to spread out from the surface of interest.
8. Our experience is based on the sol-gel immobilization on screen-printed graphite electrodes. However, the sol-gel immobilization on other supports is also possible.
9. Drying time depends on each case and should be optimized. Desiccators with or without vacuum can also be used. This step can be also performed at room temperature. However, 4°C are preferred to maintain the enzyme activity.
10. Buffer can also be used to rinse the support.
11. Although in **Subheading 2.2., item 3** PVA-SbQ with degree of polymerization: 1700 is suggested, other degrees of polymerization can be used.
12. The 50:50 ratio is suggested. However, other ratios can be used. We have observed satisfactory results with the 70:30 ratio.
13. If foam or bubbles are observed after vortex mixing, briefly centrifuge using a benchtop centrifuge.
14. Our experience is based on the immobilization on screen-printed graphite electrodes, at the bottom of Maxisorp microtiter wells and on Ultrabind modified polyethersulfone affinity membranes. However, the PVA-SbQ immobilization on other supports is also possible.

15. Exposure to ultraviolet light for 2 min at 4°C is also possible. However, exposure to neon light for 3 h at 4°C is more convenient, as it provides slower polymerization and higher reproducibility.

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8

Design of Smart Biocatalysts

Immobilization of Enzymes on Smart Polymers

Ipsita Roy and Munishwar N. Gupta

Summary

Smart polymers are water-soluble polymers that can be precipitated by an appropriate stimulus such as change of pH, ionic strength, temperature, or addition of a chemical species. Such polymers occur naturally (e.g., alginate, chitosan) but can also be synthesized chemically (e.g., methyl methacrylate polymers available commercially as Eudragit™). Linking of an enzyme to these polymers by noncovalent or covalent methods gives a biocatalyst that can be used as a homogeneous catalyst but can be recovered for possible re-use (after the reaction) by applying appropriate stimulus. The illustrative protocol shows that xylanase could be adsorbed on Eudragit L-100 and this reversibly soluble–insoluble biocatalyst could be used for hydrolysis of xylan. Interestingly, the adsorption removed cellulase impurity. This is useful for paper pulp bleaching because xylanase should be free of cellulase activity. The soluble Eudragit–xylanase conjugate could be studied by circular dichroism spectroscopy to examine conformational changes in enzymes on immobilization on Eudragit L-100.

Key Words: Smart polymers; reversibly soluble–insoluble polymers; stimuli-sensitive polymers; xylanase; paper pulp bleaching; CD spectroscopy.

1. Introduction

Smart polymers undergo dramatic changes in their solubility in response to an appropriate stimulus. As these changes are reversible, these stimuli-sensitive–stimuli-responsive polymers have also been called reversibly soluble polymers ***(1–4)***. Immobilization of enzymes on such polymers normally does not alter the smart behavior of the polymers. Therefore, such bioconjugates are called "smart biocatalysts" ***(4–6)***. Smart biocatalysts combine the virtues of both homogeneous (catalysis is carried out by the soluble form) and heterogeneous (catalyst can be

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easily recovered) catalysts. **Table 1** lists some of the smart bioconjugates described in the literature.

It is obvious from **Table 1** that smart polymers can be both synthetic as well as naturally occurring. Eudragit™ is an example of an enteric polymer that has been extensively used by many workers. In view of its original intended application, this synthetic polymer is available easily in large amounts and is fairly economical. Alginate, chitosan, and κ-carrageenan represent naturally occurring polysaccharides. All of these are fairly economical. The nontoxic nature of Eudragit, alginate, and κ-carrageenan, allow the use of smart biocatalysts based on these polymers in food processing industries.

Table 1 also illustrates the fact that in many cases noncovalent immobilization has been used for preparing such bioconjugates. In fact, in some cases, attempts to use covalent coupling methods did not succeed because noncovalent interactions dominated the adsorption of the enzyme to the matrix ***(4,7)***. In principle, all covalent coupling methods described for immobilization on solid supports ***(8)*** can be used for conjugating proteins to these polymers.

The smart biocatalysts listed in **Table 1** have been used in the bioconversion of starch, cellulose, chitin/chitosan, hydrolysis of xylan, and for obtaining protein hydrolysates. It has been observed that even with soluble matrices, the intrinsic rate of the immobilized preparation is different from that of the free enzyme. Enhanced Michaelis constant (K_m) values for the bioconjugate have been often reported ***(9,10)***. In these cases, the steric hindrance from matrix is not totally abolished, even if the latter is soluble. A possible solution to this is to use the "end-group conjugation" method ***(11,12)***. In this method, smart oligomers (instead of polymer) of *N*-isopropylacrylamide have been linked (to the enzyme) via its only one reactive end group. It was found that even with a ratio of oligomer to enzyme of 12:1, immobilized trypsin showed accessibility (toward soybean trypsin inhibitor) comparable to the free trypsin ***(12)***. Unfortunately, this conjugate showed worse stability (than even free trypsin) during thermal recycling, presumably as a result of "microenvironmental stress" ***(12)***. Smart bioconjugates, in which the polymer is linked to a specific site on the protein, have also been described ***(13,14)***. Such "site-specific conjugation" has been used for designing molecular switches ***(14)***.

As enzymes have also been used in organic solvents, it is natural that smart bioconjugates have also been evaluated in such media in terms of their re-usability and catalytic efficiency ***(15,16)***. A photosensitive bioconjugate of subtilisin gave a transesterification rate comparable to the free enzyme powder. The smart bioconjugate could be re-used four times without any significant loss of enzyme activity ***(15)***.

The smart bioconjugates, in their soluble forms, can be probed spectroscopically or by circular dichroic measurements ***(17)*** (*see* **Fig. 1**). Thus, such systems also allow workers to gain further insight into the immobilization process. It should be added that one can also immobilize enzymes on smart hydrogels. Such hydrogels are insoluble but change their volume dramatically as a result of external stimuli ***(6)***. There is a growing interest in the design and applications of such smart biocatalysts as smart biocatalysts offer no general disadvantage over enzymes immobilized on solid supports.

Table 1
Illustrative List of Smart Bioconjugates Described in the Literature to Date

Smart polymer	Protein/Enzyme	Immobilization method	Appropriate stimulus	Reference
MPM-06	Papain	Carbodiimide coupling	pH	***21***
Copolymer of *N*-isopropylacrylamide and *N*-acryloxysuccinimide or glycidyl methacrylate	IgG; alkaline phosphatase	Adsorption	Temperature and Ionic strength	***22***
N-isopropylacrylamide	Trypsin	Coupling via dicyclohexyl carbodiimide and *N*-hydroxysuccinimide	Temperature	***11***
Chitosan	Laccase	Carbodiimide coupling	pH	***23***
Eudragit S-100	Yeast lytic enzyme	Covalent coupling	pH	***1***
Eudragit L-100	Xylanase	Adsorption	pH	***17***
Alginate	Pectinase	Adsorption	Ca^{2+}	***10***
Polymer of acrylamide 1-(β-methacryloxy)-ethyl-3,3]–dimethyl-6-nitrospiro (indoline–2,2[[2*H*-1] benzopyran)	α-Chymotrypsin	Encapsulation	400 nm > λ > 300 nm	***24***
Polymerized a_{s1}-casein	Phosphoglyceromutase	Conjugation using *N*-succinimidyl 3-(2-pyridylthio)propionate	Ca^{2+}	***25***
κ-Carrageenan	Pectinase	Adsorption	K^+	***4***
κ-Carrageenan	Alcohol dehydrogenase	Covalent coupling	K^+	***26***
Hydroxypropyl methyl cellulose acetate succinate	Chitinase	Covalent coupling	pH	***27***
Poly[3-carbamoyl-1-(*p*-vinyl-benzyl)pyridinium chloride]	Trypsin	Graft polymerization	Redox couple of $Na_2S_2O_4$ and H_2O_2	***28***
Copolymer of *N*-4-phenyl-azophenyl acrylamide and *N*,*N*-dimethylacrylamide	Endoglucanase 12A	Site-specific conjugation	UV/Vis illumination	***16***
Elastin-like polypeptide (ELP)	Thioredoxin-ELP fusion protein	Adsorption	Temperature	***29***
Poly(2-hydroxymethacrylate-*co*-dimethyl aminoethyl methacrylate)	Cholesterol oxidase	Entrapment	Cholesterol	***30***

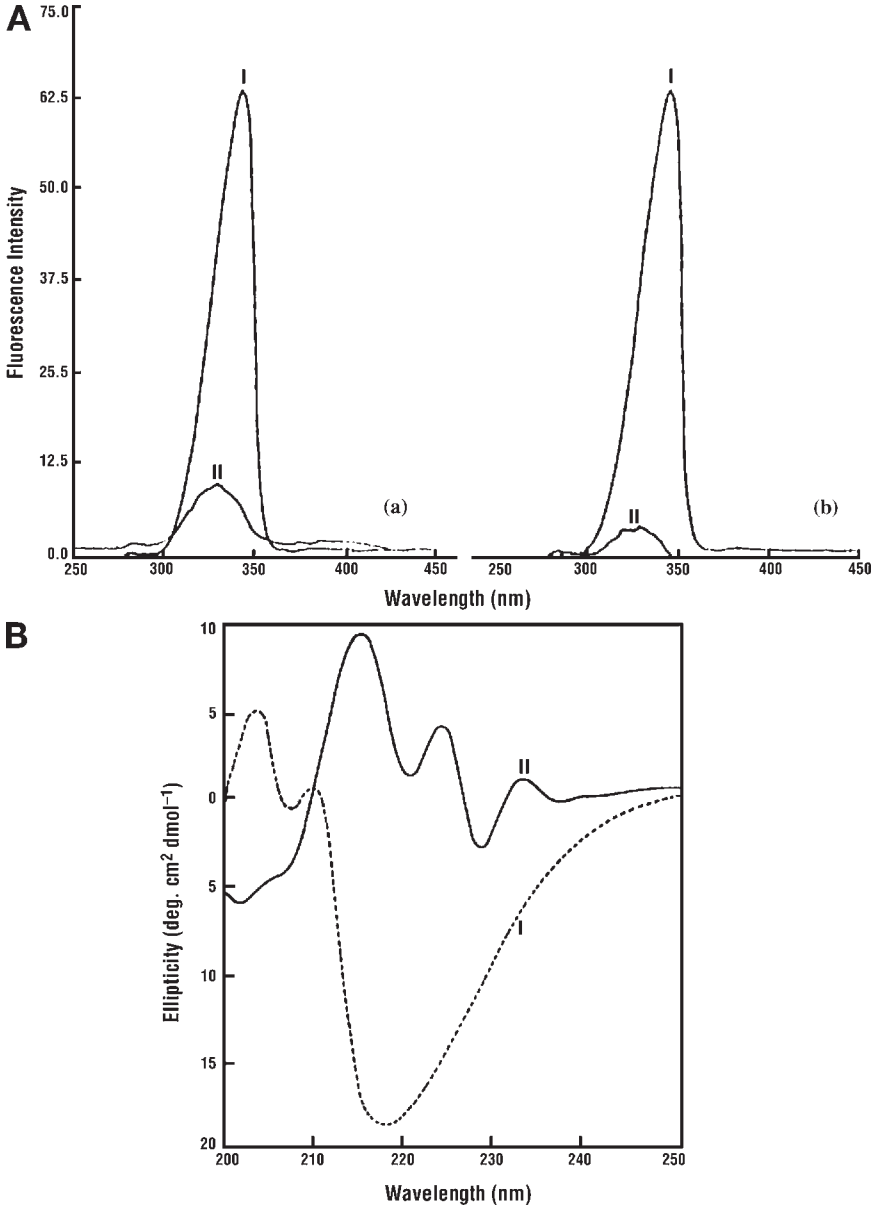

Fig. 1. Characterization of enzyme-polymer conjugate by (**A**) fluorescence spectroscopy difference spectrum of the immobilized and free enzymes and difference spectrum of the immobilized enzyme after subtracting the contribution of the polymer from the enzyme spectrum and (**B**) circular dichroic measurements. Xylanase was purified by affinity precipitation with Eudragit S-100, before being immobilized on Eudragit L-100. Equal amounts of protein were taken for (I) free as well as (II) immobilized enzyme. (From **ref. *17*** with permission from Elsevier.)

2. Materials

2.1. Assay of Xylanase Activity

1. *Aspergillus niger* xylanase (supplied as Pectinex 3XL, Novozymes, Bagsvaerd, Denmark)
2. Buffer 1: 0.05 m*M* sodium acetate buffer, pH 5.6.
3. Oat spelt xylan (Sigma Chemical Co., St. Louis, MO).
4. Dinitrosalicylic acid reagent ***(18)***.

2.2. Preparation of Eudragit L-100 Solution

1. Eudragit L-100 (Rohm Pharma GmbH, Weiterstadt, Germany).
2. 3 *M* NaOH.
3. 3 *M* HCl.
4. Distilled water.
5. Pasteur pipet.
6. Small magnetic bar.
7. Magnetic stirrer.
8. pH meter.

2.3. Immobilization of Xylanase on Eudragit L-100

1. 0.1 *N* Acetic acid.
2. Buffer 2: 0.05 *M* sodium acetate buffer, pH 4.0.
3. Benchtop centrifuge with a rotor capable of accommodating 10-mL centrifuge tubes and achieving a minimum RCF value of 12,000*g*.

3. Methods

3.1. Assay of Xylanase Activity (19)

1. Incubate the enzyme sample (1 mL, appropriately diluted in Buffer 1) in a reaction mixture containing 1% (w/v) xylan suspension (in 1 mL Buffer 1) at 50°C (*see* **Note 1**).
2. Stop the reaction after 30 min by adding 1 mL dinitrosalicylic acid reagent ***(18)*** and immersing the test tubes in a boiling water bath. Cool the test tubes after boiling for 5 min, add 10 mL of distilled water, shake, and read the absorbance of the liberated reducing sugars at 540 nm. One enzyme unit liberates 1 μmol of xylose per minute at 50°C, pH 5.6. Because the immobilized enzyme is soluble at pH 5.6, its activity can also be measured in the same manner as that for the free enzyme.

3.2. Preparation of Eudragit L-100 Solution

1. Dissolve 100 g Eudragit L-100 in 40 mL of double distilled water by stirring the suspension on a magnetic stirrer and adding 3 *M* NaOH dropwise using a Pasteur pipet until the pH increases to 11.0 (*see* **Note 2**).
2. After the polymer dissolves completely, decrease the pH to 4.3 by adding 3 *M* HCl (*see* **Fig. 2**). Centrifuge the precipitated Eudragit, decant the supernatant and dissolve the precipitate by adding 40 mL of distilled water and increasing the pH to 7.0 with 3 *M* NaOH. Make up the total volume to 50 mL with distilled water. This solution is quite stable when stored at 4°C for a few weeks.

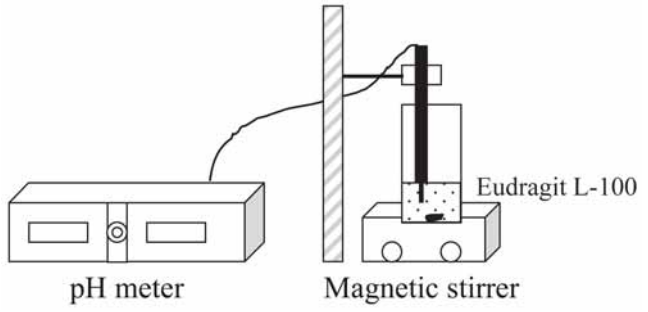

Fig. 2. Preparation of Eudragit L-100 solution for the immobilization of xylanase.

*3.3. Immobilization of Xylanase on Eudragit L-100 (17) (***see** *Note 3)*

1. Add 130 to 1070 µL of Pectinex 3XL to 2.5 mL, 2% (w/v) Eudragit L-100 solution and make up the final volume to 4 mL with Buffer 1 (*see* **Notes 4** and **5**).
2. Incubate this solution for 1 h at 25°C. After 1 h, add 130 µL of 0.1 *N*-acetic acid and let the suspension stand for 20 min (*see* **Note 6**).
3. Centrifuge the suspension at 12,000*g* for 20 min at 25°C and collect the supernatant. Wash the precipitate with 4 mL of Buffer 2 and keep for 10 min at 25°C.
4. Centrifuge the suspension as before and collect the washing. Keep on washing the precipitate until no enzyme activity is detected in the washings (*see* **Note 7**).
5. Estimate the amount of xylanase activity in the supernatant and washing.
6. Dissolve the enzyme-bound polymer precipitate in 4 mL of Buffer 1 *(see* **Note 8**) and estimate the activity of the immobilized enzyme (as described in **Subheading 3.1.**; *see* **Notes 9** and **10**).
7. In this system, the preparation obtained by starting with 200 µL of Pectinex 3XL was found to give a maximum immobilization efficiency of 0.60 ***(17)***. For characterization and applications of this conjugate *see* **ref.** ***17***.

4. Notes

1. Preincubate the enzyme, Buffer 1, and the substrate separately at 50°C for 10 min before starting the assay.
2. Initially, it is necessary to raise the pH to above 10.0 for dissolution of a fresh sample of Eudragit powder. After a clear solution has been obtained, pH 7.0 is more than adequate to dissolve a Eudragit precipitate formed at pH 4.3.
3. Eudragit is a co-polymer of methyl methacrylate and methacrylic acid. The ratio of ester to free carboxyl groups is 1:1 in the case of Eudragit L-100 and 2:1 in the case of Eudragit S-100. Often, these two show different binding affinities with a chosen protein. In the present protocol, Eudragit L-100 has been used. Eudragit S-100 could be successfully used for purification of xylanase by selective precipitation ***(17)***. This illustrates a general rule: modification of a polymer or use of related polymers may turn out to be suitable matrices for immobilization, even though the original choice may not show an adequate binding constant.

4. One of the major applications of xylanases is in paper pulp bleaching. It is therefore desirable that the xylanase being used does not contain significant cellulase activity. An interesting and useful aspect of this protocol is that cellulase activity (present in Pectinex 3XL) does not bind to the polymer and hence is not present in the immobilized form ***(17)***.
5. Another application of xylanase is in the food industry. Eudragit L-100 constitutes a safe matrix because it is a nontoxic and food-grade polymer. In fact, it is an enteric polymer.
6. This bioconjugate exploits the fact that Eudragit (and the bioconjugate) show pH-responsive reversible solubility. However, Eudragit can also be precipitated by other stimuli. Depending upon the stimulus used during noncovalent immobilization, the binding constant of the protein may change dramatically ***(20)***. This can be exploited as another parameter for obtaining the bioconjugate.
7. Irrespective of whether covalent or noncovalent immobilization is carried out, some molecules are almost always more loosely bound than other molecules of the same protein. It is therefore desirable to check the stability of the bioconjugate under process conditions (e.g., pH, ionic strength, temperature, presence of a detergent, or a substrate/product analog).
8. Presence of Eudragit lowers the pH of the solution. It is therefore necessary to adjust the pH to 5.5 with a small volume of 3 *M* NaOH to dissolve the precipitate completely.
9. The final activity expressed by the polymer-bound enzyme is critically dependent upon the polymer concentration and enzyme load at the time of preparation. This should be optimized for individual systems.
10. The thermal stability and/or pH optimum of the enzyme may change upon immobilization. The bioconjugate should be evaluated for such parameters before actual application.

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9

Affinity Immobilization of Tagged Enzymes

Silvana Andreescu, Bogdan Bucur, and Jean-Louis Marty

Summary

The development of new enzyme immobilization techniques that will not affect catalytic activity and conformation is an important research task. Affinity tags that are present or added at a specific position far from the active site in the structure of the native proteins could be used to create strong affinity bonds between the enzyme structure and a solid support functionalized with the complementary affinity ligand. The wide diversity of affinity systems creates many possibilities for enzyme immobilization. This work describes experimental strategies for affinity immobilization of tagged enzymes onto activated supports. Protocols for attaching histidine (His) and sugar-tagged enzymes onto common supports such as commercial aminated silica beads and activated graphite-cellulose are described in detail. Such strategies are generic and could be used in any application requiring immobilized biocatalysts, making future enzyme technologies more sensitive, simpler, re-usable, and less expensive. Finally, strategies for removing nonspecific adsorption and examples of possible applications of these methods to enzyme sensors are discussed.

Key Words: Tagged enzymes; affinity immobilization; histidine; NTA; glycoenzyme; concavalin A.

1. Introduction

In recent years, advances in immobilization techniques have remarkably influenced the design and performance of a variety of enzymatic systems ranging from bioreactors to an important number of biosensor devices. Immobilization of enzymes is of significant importance in many practical applications. In immobilized states enzymes are more stable and can also be re-used, thereby considerably reducing costs associated with immobilization. Many efforts have been focused on finding an immobilization procedure that maintains the greatest characteristics of the enzymatic system. There are several requirements for selecting the immobilization method: (1) to preserve the enzyme activity and keep it as close as pos-

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sible to its native state, (2) to ensure stability of the biomolecule, (3) to be low cost, and (4) to have sufficient sensitivity and selectivity for the analyte of interest. In addition, an appropriate selection is dictated by the type and nature of the enzyme and/or the support, as well as the final application of the system. Various methods such as adsorption, covalent binding using bifunctional reagents ***(1)***, physical entrapment in a polymer matrix ***(2)***, self-assembled monolayers (SAM), ***(3)*** or Langmuir-Blodgett films ***(4)*** have been used to immobilize enzymes onto different surfaces. Because the overall performances of the devices based on immobilized proteins are strongly affected by this process, intensive efforts need to be maintained in order to develop successful immobilization procedures.

A new trend in enzyme immobilization is to create different (bio)affinity bonds between an "activated" support and a specific group present on the protein surface. In this case, enzyme immobilization is achieved by using an affinity tail at a specific position of the protein sequence, which does not affect the activity or the folding of the protein. A simple binding of this modified enzyme is then performed onto a support. This procedure offers several advantages including: (1) selective and oriented immobilization similar for all biomolecules because of the same connection "binding site or specific group of the support" (in the case that only an affinity tail is presented on the protein surface), (2) the minimization of the conformational changes induced in the enzyme structure, and (3) the possibility of reloading the same activated surface with enzyme and to re-use the support according to different demands. There are two main requirements to immobilize an enzyme by specific affinity interactions: (1) the choice of a biocompatible support that should either possess the necessary functional groups (with very high affinity) and be able to specifically recognize a target group present on the external surface of the protein of interest or that can be easily functionalized and (2) the availability of a specific groups on the surface of the enzyme.

The choice of the biocompatible supporting material is essential. In most cases, the native material does not meet all the needs of enzyme immobilization and therefore requires further functionalization steps. For this reason it is necessary to identify and/or to introduce different start linking groups onto the support surface (e.g., -NH_2, -COOH, -OH, …) onto which a "smart" linker can be connected. These linkers are selected to posses a high affinity for a specific group (tag) of the enzyme. They may be designed as sandwich-like structures (support-specific linker-enzyme), usually realized layer by layer in successive independent steps separated by washing of excess activation agents. This permits the optimization and tuning of the entire immobilization protocol so that the protein is strongly bound onto the support at a specific point while avoiding the denaturation processes of the enzyme. Several commercial preactivated membranes are available and can be used as supports to immobilize enzymes using this method.

Affinity tags such as histidine (His), cysteine (Cys), or mannose-binding proteins can participate in the affinity binding ***(5–9)***. However, even though many enzymes contain such residues in their amino acid sequences, their number is very limited; very few of them are located on the protein surface ***(5)*** and are accessible for binding to the support. Moreover, genetic engineering methods permit the production of tagged proteins by attaching an affinity tag to amino or carboxyl terminals far enough away from the active site to reduce the steric hindrance during the

biocatalytic processes and extend the application field of this type of immobilization to enzymes lacking the required target group on their sequence ***(10,11)***.

This chapter proposes and discusses the development of two affinity approaches for the immobilization of tagged enzymes: one based on metal chelate affinity (MCA) and one based on concanavalin A (Con A) ***(12–15)***.

2. Materials

2.1. Acetyl Choline Esterase Immobilization Using His-Tag and Metal Chelate Affinity

1. 0.1 *M* Phosphate buffer solution (PBS), pH 7.0.
2. Acetyl choline esterase (AchE) from *Drosophila melanogaster (*GTP Technology, Toulouse, France*): wild type* and recombinant AChE-$(His)_6$. (AChE-WT is a soluble dimeric form deleted from a hydrophobic peptide at the C-terminal end from *D. melanogaster* AChE. AChE-$(His)_6$ originates from AChE-WT with a 3x histidine tag replacing the loop from amino-acids 103 to 136).
3. Epibromohydrin 98% (Sigma). **Caution:** highly toxic and corrosive.
4. 4 *M* NaOH solution.
5. $NiSO_4 \cdot 6H_2O$ (Aldrich).
6. Metal chelate: a chemically derivative of the nitrilotriacetic acid (NTA)—the *N*-(5-amino-1-carboxypentyl) iminodiacetic acid—synthesized from *N*-benzyloxycarbonyl-L-lysine according to the procedure described by Hochuli et al., 1987 ***(6)***.
7. 1.5-mL Eppendorf microtubes; Pasteur pipets and bulbs.
8. Microcentrifuge (Denver Instrument Company) suitable for 1.5-mL Eppendorf microtubes to centrifuge the mixture of silica-NTA-Ni with the enzyme solution.
9. 5-mL Graduated plastic syringe.
10. Hydroxyethyl-cellulose (HEC) (Fluka).
11. Graphite powder (Timcal).
12. 0.1 *M* Phosphate washing buffer solution, pH 7.5, supplemented with 0.1% Tween-20.
13. 0.2 *M* NaCl and 2 *M* $MgSO_4$ to eliminate electrostatic and hydrophobic nonspecific interactions.
14. Bovine serum albumin (fraction V, Sigma) 0.5% (w/v) prepared in PBS to saturate the adsorption binding sites of the protein.
15. Small magnetic stir bar. Magnetic stirrer types AM 3000 D (Bioblock, France) and a silicon oil bath with temperature control.
16. Buchner flask, glass filter, and suction filter pump.

2.2. Immobilization of Glycoproteins Based on Con A Affinity—Acetyl Choline Esterase Immobilization on Aminated Silica Beads

1. 0.1 *M* PBS solution, pH 7.0, was supplemented with 0.1 *M* 0.1 *M* $CaCl_2$, and 0.1 *M* $MnCl_2$.
2. 0.1 *M* Carbonate buffer solution, pH 11.0.
3. 1 mg/mL Working solutions PBS Con A extracted from Jack Bean (*Canavalia ensiformis*; *see* **Note 1**).

4. AChE (E.C. 3.1.1.7) type V-S Electric eel (Sigma; *see* **Note 2**).
5. Methyl α-D-mannopyranoside (Sigma).
6. Divinyl sulfone (DVS) Fluka.
7. Amino functionalized silica beads with a 25- to 40-mm diameter LiChroprep® NH_2 (Merck) were used as immobilization support.
8. Acetylthiocholine chloride ATCh (Sigma) stock solution was prepared daily in water (*see* **Note 3**).
9. 1.5-mL Eppendorf microtubes were shaken with a flask shaker SF1 (Stuart®, Barloworld, Stone, UK); set at speed 4.
10. Minicentrifuge Capsule HF-120 (Tomy, Kogyo, Japan) was used to separate the beads from reaction media.
11. A vortex ReAX 2000, (Heildolph, Schwabach, Germany) was used to mix the Eppendorf microtubes during washing cycles.

3. Methods

3.1. Immobilization Using His-Tag and Metal Chelate Affinity

*3.1.1. Functionalization of the Graphite/Hydroxyethyl–Cellulose Mixture With the Nitriloacetic Acid–Ni and Immobilization of Acetyl Choline Esterase-$(His)_6$ (*see ***Note 4***).*

1. Synthesize the NTA chelate [an NTA derivative: *N*-(5-amino-1-carboxypentyl) iminodiacetic] from *N*-benzyloxycarbonyl-L-lysine according to the procedure described by Hochuli et al. 1987; ***(6)***.
2. Check the chelation properties of the synthesized NTA and the affinity binding capacity of the AChE-$(His)_6$ for a sepharose column loaded with NTA and complexed with Ni ions. Use the same procedure described by Hochuli et al. (1987; ***6***) to load the sepharose with the NTA and Ni and to equilibrate the column. A plastic syringe was packed with the sepharose-NTA-Ni.
3. Deposit 35 U AChE-$(His)_6$ in PBS onto the sepharose column and add PBS. Collect fractions of 1.5 mL and measure the enzymatic activity in each fraction to determine the amount of the immobilized enzyme.
4. A modified procedure is used to functionalize the graphite/HEC (*see* **Fig. 1** and **Note 4**) with the synthesized NTA and then with Ni ***(10,11)*** (*see* **Note 5**).
5. Activation of the hydroxyls groups of the graphite/HEC mixture: in a 10-mL glass flask add 0.2 g graphite, 0.1 g HEC, and 2 mL epibromohydrine. While stirring, add 4 mL 4% NaOH. Stir the solution for 4 h under controlled temperature conditions (30°C).
6. Wash intensively with water until a neutral pH is achieved in order to eliminate the excess of epibromohydrin and NaOH. Separate the water by centrifuge and filter.
7. Loading with the NTA derivative: in a round bottom flask containing the resulting activated graphite/HEC add 0.1 g NTA derivative, 0.2 g Na_2CO_3, and 1 mL distilled water. Stir the mixture overnight at 60°C. Wash with water to remove the excess of NTA. Remove the water first by centrifuge and then filter.
8. Complexation with Ni: wash with a 1% (w/v) Ni^{2+} solution.

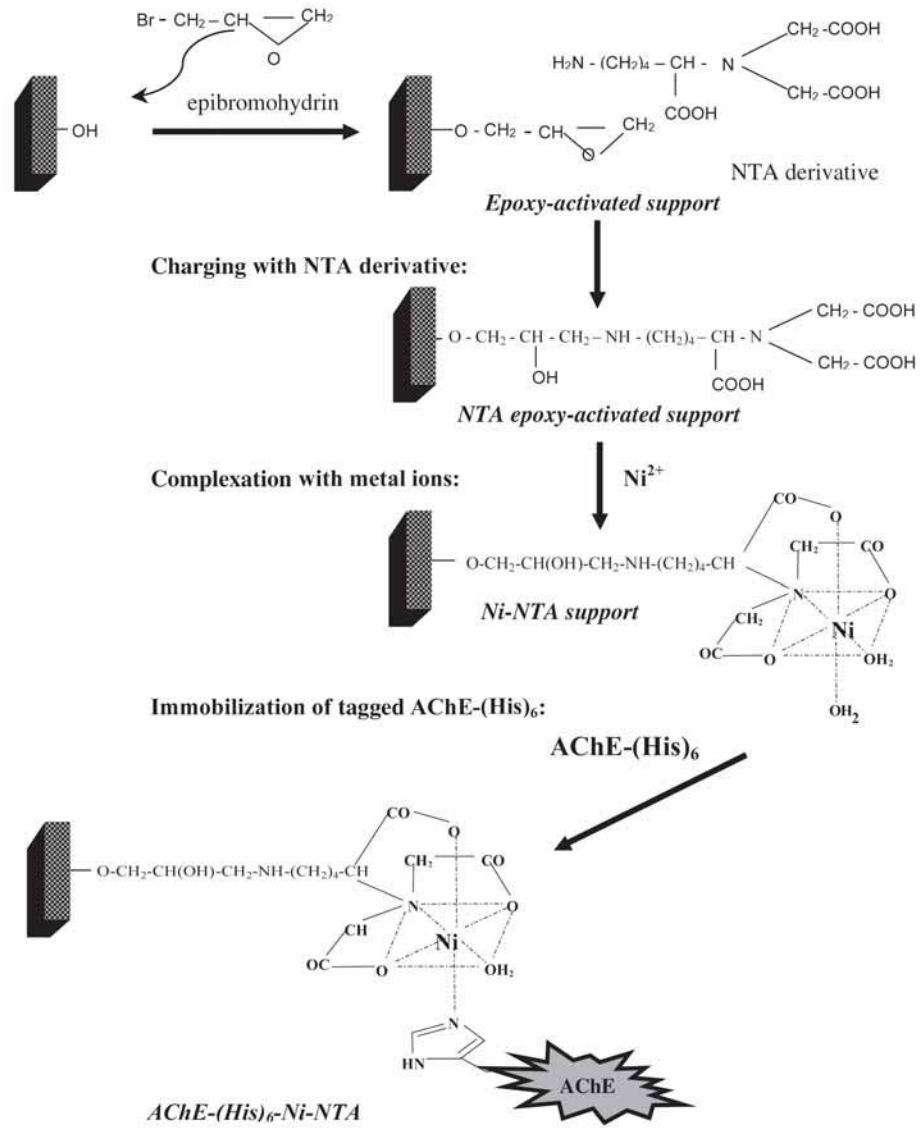

Fig. 1. Strategy for the functionalization of the support with a NTA-Ni chelate and immobilization of a His-tagged AChE.

9. Eliminate the excess Ni by washing with distilled water.
10. Wash with 0.1 *M* phosphate washing buffer, pH 7.5, containing Tween-20, NaCl, and $MgSO_4$ to minimize the nonspecific interactions.
11. The resulting graphite/HEC–NTA–Ni can be used for immobilization of a tagged enzyme by MCA ***(11)***. The resulting functionalized support can be employed to

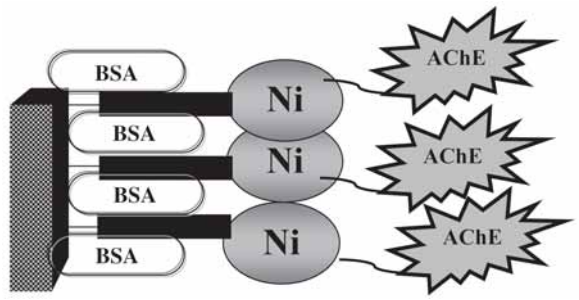

Fig. 2. Schematic diagram of the surface with immobilized tagged enzyme via metal chelate affinity.

construct an enzyme biosensor on which any His-tagged enzyme can be immobilized (*see* **Fig. 2**).

12. Add a solution of 0.5% (w/x) BSA to saturate the binding sites of the support accessible to enzyme adsorption.
13. Deposit a solution of His-tagged enzyme prepared in PBS, pH 7.0 (*see* **Note 6**), of the desired activity function of the final application of the enzyme system.

3.1.2. Evaluation of Nonspecific Interactions and Re-Usability of the Support

1. Perform the same procedure to immobilize a wild type nontagged enzyme (the same amount as used for the His tagged) onto a similar functionalized support.
2. Check to see if there is any enzyme bounded onto the support. This corresponds to the enzyme fixed by adsorption.
3. Immobilization of His-tagged enzymes by affinity via metal chelate is fully reversible upon addition of competitive compounds such as imidazole (Im) or upon removal of metal ion from the NTA group with stronger chelators such ethylene diamine tetraacetic acid (EDTA). Simple washing with a solution of Im or EDTA regenerates the support (*see* **Note 7**).
4. Eliminate the enzyme bound by affinity by simply washing with a 0.1 *M* imidazole solution (*see* **Note 8**).
5. Eliminate the Ni ions with a solution of 0.1 *M* EDTA (*see* **Note 9**).

3.2. Immobilization of Glycoproteins Based on Con A Affinity—Application to Acetyl Choline Esterase Immobilization on Aminated Silica Beads

The immobilization procedures were performed in a few independent steps starting with the activation of the support. The immobilization was carried out with 10 mg of silica beads used as support in 1 mL of PBS or carbonate buffers to ensure the required pH value of the reagents or biocomponents used for each step. For each phase of the protocol the reactions were performed in 1.5-mL Eppendorf microtubes, mechanically shaken for 2 h at speed 4. The removal of the reagents from the reaction media was accomplished by four washing cycles, each one con-

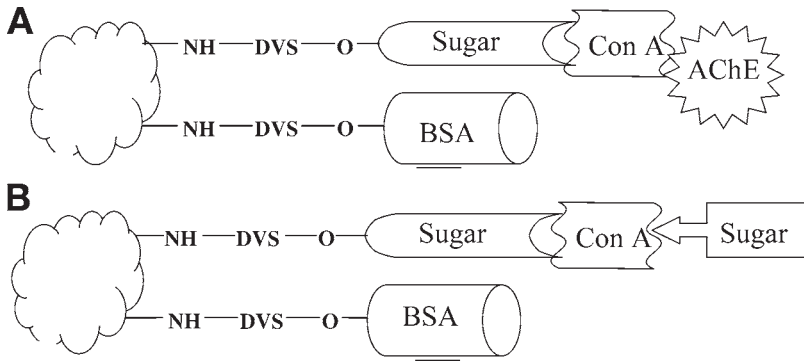

Fig. 3. Scheme of the immobilization of the enzymes by affinity via Con A: the sandwich structure of the immobilization protocol (**A**) and the blank with the lectin block by methyl α-D-mannopyranoside represented by sugar (**B**).

sisting of: reaction media centrifugation, supernatant removal, vortex stirring with 1 mL of the buffer for 1 min (*see* **Note 10**). If the buffer had to be changed between steps then the first buffer was used for the first two cycles and the new buffer in the final two washing steps.

1. Activation of the amino groups of the support was performed by stirring the beads in 1-mL carbonate buffer containing 10% DVS (v/v).
2. The activated beads were allowed to react with the carbohydrate (methyl α-D-mannopyranoside) in 1 mL of 10% DVS solution (*see* **Fig. 3A)**.
3. The nonreacted DVS-activated amino groups and adsorption sites were blocked with 1 mL BSA (10% m/v in carbonate buffer).
4. 1 mg Con A dissolved in 1 mL PBS was added over the beads and the lectine was immobilized on the bounded sugar via affinity links.
5. Various enzyme quantities (0.3–3.3 U AChE) were immobilized on the support through a biorecognition mechanism.
6. In order to eliminate the nonspecific adsorption phenomena a final washing was performed after enzyme immobilization for 12 h by stirring the beads in 20 mL PBS supplemented with 1 *M* KCl, at 4°C ***(16)***.
7. The nonspecific AChE adsorption was evaluated by replicating the immobilization protocol, but this time the lectine was blocked with 1 mL of 10% methyl α-D-mannopyranoside in PBS before contact with enzyme (*see* **Fig. 3B)**. Also, the enzyme was dissolved in PBS which also contained 10% of methyl α-D-mannopyranoside in order to ensure continuous competition between glycoproteine and sugar for the lectine from the support.

3.3. Application of the Affinity Immobilization Methods to the Construction of a Screen-Printed Biosensor Sevice

1. The amperometric biosensors were fabricated in our laboratory using the screen-printed technology. The procedure consists of successive deposition of several layers onto a plastic support ***(17)***.

2. Then, according to each affinity immobilization procedure, a specific composite paste with or without enzyme was deposited onto the surface of the working electrode surface. For this specific application the working electrode was used as a basic support for the final immobilization of the enzyme.

3.4. Biosensor With the Enzyme Immobilized Via Affinity on the Graphite/Hydroxyethyl–Cellulose–Nitriloacetic Acid–Ni

1. Mix the Graphite–NTA–Ni with graphite-TCNQ powder ***(18)*** (10%) and 1 mL 4% (w/v) HEC.
2. Stir the mixture for 3 h to ensure paste homogeneity.
3. Print the working paste over the working electrode surface using the screen-printed technique.
4. Store at 4°C in dry state under vacuum.
5. Deposit 2 µL of a 0.5% (w/v) BSA solution.
6. Deposit 2 µL of His-tagged enzyme solution at the desired activity before the use of the electrode.
7. Prepare blank electrodes with the same amount of wild enzyme to evaluate the nonspecific adsorption and to confirm the specificity of the affinity binding.
8. Rinse the electrode with the washing buffer before use.

3.5. Biosensor With the Enzyme Immobilized Via Con A

1. Mixed the aminated beads with immobilized enzyme with a paste containing 150 mg graphite-TCNQ in 1 mL 4% (w/v) HEC solution (*see* **Note 11**).
2. Deposit manually and gently spread 1µL of this paste onto the surface of working electrode.
3. Dry the electrodes for 1 h in ambient atmosphere and then for 2 h in a dessicator under a slight vacuum.
4. Store the electrodes in sealed plastic bags at +4°C ***(16)***.

4. Notes

1. The Con A solutions were prepared in PBS, 6 h prior to use to allow the reactivation of the denatured lectin by Ca^{2+} and Mn^{2+} ***(19)***.
2. AChE was dissolved in PBS. Small aliquots were stored until first use at –18°C and after at +4°C. AChE activity was spectrometrically ***(20)*** measured using ATCh and Ellman reagent (λ = 412) each time before being used.
3. The measurement of the enzyme activity has to be carried out immediately after the addition of the substrate.
4. HEC was used as additional sources of OH groups.
5. The procedure can be adapted to any support containing OH groups exposed to the surface and able to bind to the metal chelate.
6. The pH is an important parameter for the affinity interactions and strongly influences the binding efficiency. These interactions have been shown to be stable at neutral pH ***(21)***. A pH between 4.0 and –6.0 removes the tagged proteins and this principle can be used to regenerate the support.
7. It is important to notice that these procedures regenerate only the functionalized support and not the enzyme.

8. By competition with imidazole, the enzyme fixed by affinity is removed from the support.
9. Regeneration with EDTA is less desirable since the Ni ions will elute with the enzyme and an additional use of the support involves another charging with nickel and then with the enzyme.
10. The efficiency of the washing was tested at the separation of beads with immobilized AChE from 1 mL of ATCh 1 *M* and no color obtained with an Ellman's reagent test.
11. The HEC solution was prepared by stirring for few hours in distilled water with a magnetic bar until a homogeneous, viscous solution was obtained. The appropriate quantity of graphite–TCNQ was added to the resulting HEC, followed by stirring for a few additional hours. The mixture was kept at +4°C.

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10

Bioaffinity Immobilization

Ipsita Roy and Munishwar N. Gupta

Summary

In bioaffinity immobilization, the enzyme/protein is immobilized via bioaffinity interactions. A large number of affinity pairs such as lectin–sugar, antigen–antibody, and biotin–avidin are known. The use of affinity tags to create fusion proteins that can bind to the desired matrix expands the list further and leads to a more or less a general method. Two versions of bioaffinity immobilization are possible. In the first, the matrix is precoupled to an affinity ligand and the target enzyme is added. In the second, the enzyme is conjugated to another molecule that in turn has affinity toward a matrix. The bioconjugate of the enzyme can be obtained either by chemical cross-linking preparation or as a fusion protein. The concept of bioaffinity immobilization is illustrated with a protocol that utilizes the second approach. A conjugate of β-galactosidase with lectin, concanavalin A, was prepared by cross-linking with glutaraldehyde. The conjugate was bound to Sephadex G-50. The protocol was originally developed for creating an immobilized biocatalyst for lactose hydrolysis. Lactose hydrolysis is biotechnologically relevant for whey hydrolysis and for producing low-lactose milk (for consumption by lactose intolerant persons)

Key Words: Reversible immobilization; lectin bioconjugates; β-galactosidase; low-lactose milk; whey hydrolysis; lactose hydrolysis; fusion proteins; Con A.

1. Introduction

In bioaffinity immobilization, the protein/enzyme is linked to a matrix via biospecific interactions. There are two strategies that are followed for bioaffinity immobilization, which are outlined in **Table 1** and illustrated in **Fig. 1**. In either case, an affinity pair is involved. In principle, all affinity pairs (affinity ligand–target protein) used in affinity chromatography ***(1)*** can also be used for affinity immobilization. However, in affinity chromatography the design of the protocol is geared toward ensuring adequate dissociation (of the target protein), whereas in affinity immobilization the immobilized molecule does not come off the matrix.

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Table 1
Strategies Involved in the Immobilization of Enzymes/Proteins on a Matrix Via Bioaffinity Interactions

Strategy	Procedure	Comments
A.	The matrix is activated and precoupled with an affinity ligand. The target enzyme is added.	The enzyme is not subjected to harsh conditions. If the affinity ligand is a substrate/product ana logue, the chances of enzyme dissociating from the affinity ligand are high.
B.	The enzyme is conjugated to another molecule, which in turn has affinity toward a matrix. The bioconjugate can be prepared by chemical cross-linking or obtained as a fusion protein.	A more generic approach is possible for preparing a series of immobilized enzymes without varying the affinity pair.

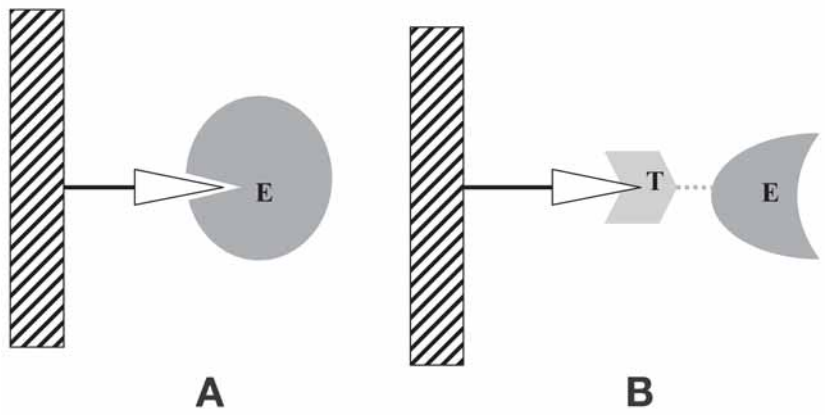

Fig. 1. Two commonly used strategies for bioaffinity immobilization of proteins. (**A**) The enzyme (E) has affinity for an affinity ligand (-β) linked to the matrix. (**B**) The enzyme (E) is linked to a fusion tag (T), which has affinity for an affinity ligand (-β) linked to the matrix.

This adherence can be achieved by choosing affinity pairs that have relatively higher association constants, and/or operational conditions favoring zero leaching. Mattiasson ***(2)*** wrote an excellent introduction to the method and listed association constants of some frequently used affinity pairs. More recently, Saleemuddin ***(3)*** has reviewed this approach with a focus on affinity immobilization on monoclonal/polyclonal antibodies and lectins. A review with a sharper focus on the use of concanavalin A (Con A)-based supports is also available ***(4)***. It should be mentioned that the classical view of the affinity of biological molecules

has now been replaced with a broader concept, according to which a ligand may not have any biological relationship with the target protein, either in vivo or in vitro ***(5)***. Thus, a dye, a metal ion complex, or a peptide may all be useful affinity ligands ***(6–8)***. In many cases, this provides a successful alternative to more costly affinity ligands such as lectins and monoclonals, although the availability of efficient bioseparation protocols should result in lowering the cost of affinity ligands like lectins and antibodies ***(1)***.

The precoupling of affinity ligand to a matrix can be carried out with any covalent coupling method that is generally used for obtaining affinity media ***(9,10)***. If the affinity ligand itself is a biologically active protein (e.g., an antibody or a lectin), the design of this precoupling step has to be such that optimum accessibility of binding sites on this affinity ligand (in the precoupled form) to the enzyme (to be immobilized) is possible. Thus, oriented immobilization of proteins as affinity ligands is aimed at linking such affinity ligands (to the matrix) via a site that leaves the binding site free. For example, linking antibodies via F_c portion leaves their binding sites free for interacting with enzymes ***(11)***. A recent work uses site-specific attachment by use of site-directed mutagenesis as another approach to attach the protein affinity ligand in such a way that its binding site is free for "affinity recognition" of the enzyme ***(12)***. Biotin–avidin or biotin–streptavidin technology ***(13)*** is now well developed. If the matrix is labeled with avidin/streptavidin or biotin, the enzyme tagged with biotin or avidin/streptavidin, respectively, would allow bioaffinity immobilization with high binding constants ***(12)***. The linking of the enzyme with one of the members of the affinity pair can also be done by chemical cross-linking ***(14)***. Alternatively, fusion proteins can be obtained by recombinant methods ***(15,16)***.

"Affinity layering" is a relatively recent approach in bioaffinity immobilization ***(17,18)***. This interesting approach allows large amounts of glycoenzymes to be immobilized by creating alternate layers of Con A and the glycoenzyme. Gemeiner et al. ***(19)*** described a "superactive" immobilized invertase by a somewhat similar approach.

Immobilization of various enzymes on Con A-derivatized supports has been reported to significantly enhance their stability under various denaturing conditions, during storage, and even for long-term continuous operation ***(20)***.

The use of fusion proteins opens several exciting possibilities in the context of bioaffinity immobilization. Use of histidine-rich affinity tails to immobilize enzymes on immobilized metal affinity supports ***(21)*** or chimeric enzymes with cellulose-binding domains ***(22,23)*** are two well-known examples of this generic approach. A recent work uses a histidine-tagged fragment of protein A, which showed high affinity for IgG ***(24)***.

The proteins/enzymes immobilized by bioaffnity immobilization have, more often, been used for bioanalysis. The protocol ***(25)*** given in this chapter illustrates the application of the approach in bioconversion. **Table 2** catalogs other applications. Anspach and Altmann-Hasse ***(26)*** have described the immobilization of *Escherichia coli* penicillin G aminohydrolase on immobilized metal chelate supports. The fusion protein consisting of Protein A and β-lactamase immobilized on IgG-Sepharose was found to hydrolyze penicillin G more efficiently than the covalently immobilized enzyme ***(27)***. Similarly, the immobilization of cellulose-

Table 2
Applications of Enzymes/Proteins Immobilized Through Affinity Interactions

Affinity ligand	Enzyme/protein immobilized	Application	Ref.
Con A-Sepharose	Cellobiose	Hydrolysis of cellobiose	31
Cellulose	Fusion protein of CBD-β-galactosidase	Hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside	28
CBD	Glucose oxidase	Glucose biosensor during fed-batch fermentation of *E. coli*	32
Human serum albumin coupled to Con A	Peroxidase	Biosensor for measurement of hydrogen peroxide	33
IgG	Protein G β-2 domain	Fibre optic assay for amplified DNA products	34
Concanavalin A	Submitochondrial particles	Continuous catalytic transformations involving succinate-cytochrome *c* reductase	35
Monoclonal antibody epitope at the C-terminus of C5a receptor	C5a receptor	Possible application in combinatorial library screening and receptor characterization studies	36
Concanavalin A	Invertase	Amplification of flow microcalorimetry signal	19
Carbohydrate epitope of ganglioside	Anti-ganglioside monoclonal antibodies	Determination of binding constants	37
Co^{3+}	Concanavalin A	Coupled system of invertase immobilized on the matrix with glucose oxidase bound directly via Cu^{2+} for direct measurement of glucose concentration	38
Polyclonal antibodies	Glucose oxidase and peroxidase	Flow injection analysis for measuring glucose and hydrogen peroxide	18
Protein A	Anti-glutathione-*S*-transferase (GST) monoclonal antibody	Synthesis of regenerable surface for immunosensor application	39
Avidin	Biotinylated substrate	Fabrication of protein micropatterns	40
Photoisomerizable antigen layer	Antibody	Photolithography	41
Ni^{2+}	His_6-lactate dehydrogenase	Bioelectrode for monitoring conversion of lactate to pyruvate	42

binding domain (CBD)-β-galactosidase fusion protein on cellulosic supports resulted in a fairly efficient reusable biocatalyst ***(28)***.

Recently, combinatorial peptide synthesis has made it possible to obtain peptides as affinity ligands with a predetermined activity ***(5)***. Such peptide affinity ligands, in principle, may also be used for affinity immobilization of enzymes since it will be possible to screen such ligands for binding constants in the range desirable for affinity immobilization. In this context, Hahn et al. ***(29)*** have outlined an innovative approach wherein such peptide ligands are linked only to accessible pore sites and do not occupy internal space of the matrix pores. This results in an affinity matrix with better binding capacity and ligand utilization for the target protein.

It is obvious that several innovative variants of bioaffinity immobilization are available. Hence, today, bioaffinity immobilization is a viable and good choice as far as immobilization strategies are concerned.

2. Materials

2.1. Assay of β-Galactosidase Activity

1. *E. coli* β-galactosidase (Sigma Chemical Co., St. Louis, MO)
2. Buffer 1: 100 m*M* sodium phosphate buffer, pH 6.5.
3. *o*-Nitrophenyl-β-D-galactopyranoside (ONGP; CSIR Centre for Biochemicals, New Delhi, India).
4. Buffer 2: 300 m*M* sodium phosphate buffer, pH 7.5, containing 3 m*M* $MgCl_2$
5. Buffer 3: 10 m*M* Tris acetate buffer, pH 7.5, containing 10 m*M* $MgCl_2$.
6. Double distilled water.
7. 1*M* β-Mercaptoethanol (Fluka, St. Louis, MO). Solution from the bottle is diluted with double distilled water.
8. 1 *M* Na_2CO_3.
9. Bausch and Lomb colorimeter.

2.2. Preparation of Con A-β-Galactosidase Conjugate and Immobilization on Sephadex G-50

1. Concanavalin A (Con A; CSIR Centre for Biochemicals, New Delhi, India).
2. 25% Glutaraldehyde solution (Reidel-Haan, St. Louis, MO). Dilute with double distilled water, as required (*see* **Note 1**).
3. Vortex mixer.
4. Sephadex G-50 gel (Pharmacia, Uppsala, Sweden). The gel is swollen and deaerated per the manufacturer's instructions (Handbook of Amersham Biosciences: Gel Filtration: Principles and Methods).
5. Frac 100 fraction collector (Pharmacia, Uppsala, Sweden)

3. Methods

3.1. Assay of β-Galactosidase Activity (30)

1. Incubate the enzyme sample (200 µL in Buffer 1) in a reaction mixture containing 1.50 mL Buffer 2, 1.65 mL double distilled water, 0.014 *M* ONGP solution in 0.45 mL of Buffer 3), and 1 *M* β-mercaptoethanol in 0.75 mL double distilled water at 25°C.

2. Stop the reaction after 5 min by adding 4 mL 1 *M* Na_2CO_3.
3. Read the absorbance of the liberated *o*-nitrophenol at 405 nm. One enzyme unit liberates 1 µmol of *o*-nitrophenol per min at 25°C, pH 6.5.
4. For estimating the enzyme activity of the Sephadex-bound enzyme, the enzyme sample is pipeted with a precision air displacement pipet. The end of the tip is cut and the conjugate is taken in 50% liquid phase (Buffer 1) to facilitate pipeting (i.e., 100 µL of enzyme-bound beads and 100 µL Buffer 1 is incubated with the substrate, shaken constantly, and assayed as above).
5. Before reading the absorbance of the solution, centrifuge at 5000*g* for 5 min so that the matrix settles down.

3.2. Preparation of Con A-β-Galactosidase Conjugate and Immobilization on Sephadex Matrix

1. Prepare solutions of 4 mg/mL Con A and 1 mg/mL β-galactosidase separately in Buffer 1 containing 500 µL 1 *M* NaCl. Mix Con A and 100 µL β-galactosidase by gentle shaking in a vortex mixer and cool to 4°C (*see* **Note 2**).
2. Slowly add 20 µL of a cold 25% glutaraldehyde solution with constant mixing (*see* **Note 3**). Allow the mixture to stand at 4°C for 30 min (*see* **Note 4**).
3. Wash 10 mL (settled volume) of Sephadex G-50 gel in a sintered glass funnel connected to a Buchner funnel, under mild suction.
4. Wash the gel thoroughly with double distilled water (5 portions of 30 mL each).
5. Transfer this moist gel cake to a beaker and add sufficient volume of Buffer 1 so that the gel forms a thick slurry.
6. Transfer this slurry to a glass column (1 × 15 cm) using a glass rod aligned diagonally across the column (*see* **Fig. 2**) so that the gel is transferred to the column in one smooth motion (*see* **Note 5**).
7. Wash the packed bed of gel with 5 bed volumes of Buffer 1.
8. Load the enzyme–conjugate mixture directly on the column. Elute the unbound components with Buffer 1 containing 0.1 *M* NaCl at 22 mL/h (*see* **Note 6**).

4. Notes

1. Glutaraldehyde polymerizes in solution. Its cross-linking efficiency is, in fact, dependent upon the existence of oligomers. Hence, old preparations give better cross-linking results. Equally important, use the same glutaraldehyde preparation for consistent results.
2. Conjugation at pH 7.0 leads to precipitation of β-galactosidase. This is the reason why pH 6.5 has been chosen for intermolecular cross-linking.
3. A large number of cross-linking reagents are available. Some of these may turn out to be more efficient in terms of conjugation efficiency and are likely to give more consistent results. However, glutaraldehyde was used (and continues to be used) as a cross-linking reagent by many researchers because it is readily available and turns out to be quite economical.
4. If the amount of β-galactosidase is increased to 200 µg and the cross-linking is continued for 30 min, the protein precipitates. In this case, better results are obtained by limiting the cross-linking time to 15 min.

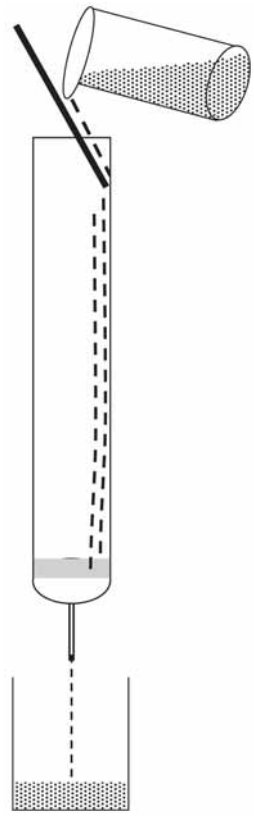

Fig. 2. Packing of a column with Sephadex G-50 gel for bioaffinity immobilization of Con A–β-galactosidase conjugate.

5. This is required so that no air bubbles get trapped in the gel matrix.
6. In some applications, it is possible that the cost of the matrix is comparable to the enzyme. In many such cases, it may be desirable to strip the enzyme/conjugate off the matrix and reload the fresh lot of enzyme/conjugate on the matrix.
7. It is necessary to check that the affinity ligand is also stable under operational conditions. Here, 50°C has been used as the process temperature, where Con A is known to be stable ***(25)***.
8. Immobilization, often (but not always), enhances thermal stability. Thus, it may be possible to work at higher temperatures provided the affinity ligand is also stable *(see* **Note 7**). Similarly, it is necessary to check the optimum pH of the immobilized enzyme before deciding the process parameters.
9. In some designs, it is possible that the product causes dissociation of the enzyme/enzyme conjugate from the matrix. However, suitable reactor design such as packed bed/fluidized bed reactors may avoid local high concentration of the product to that level.

Acknowledgments

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11

One-Step Purification, Immobilization, and Stabilization of Poly-Histidine-Tagged Enzymes Using Metal Chelate–Epoxy Supports

Cesar Mateo, Benevides C. C. Pessela, Valeria Grazu, Rodrigo Torres, Fernando López-Gallego, Jose M. Guisan, and Roberto Fernandez-Lafuente

Summary

In this chapter, the combined use of the selectivity of metal chelate affinity chromatography with the capacity of epoxy supports to immobilize poly-His-tagged proteins via multipoint covalent attachment is shown. This has been achieved by designing tailor-made chelate–epoxy supports. In order to selectively adsorb the poly-His-tagged proteins, a very small density of Me-chelate groups was introduced in the epoxy supports. This allows for the retention of most of the epoxy groups available to multipointly react with the proteins. To reach the maximum support–enzyme reaction, after the first covalent immobilization process, the derivatives were incubated at alkaline pH conditions—where the nucleophile groups of the protein are more reactive. This protocol allows for the creation of immobilized preparations containing almost pure poly-His-tagged enzymes, even when using crude extracts, and enzymes much more stable than soluble ones.

Key Words: Tailor-made IMAC supports; heterofunctional supports; selective adsorption of enzymes; oriented immobilization; enzyme stabilization by multipoint covalent attachment.

1. Introduction

The use of pure enzyme to prepare immobilized enzyme derivatives may be very relevant in biocatalysis. This may allow for improvement of the volumetric activity of the support (the only immobilized enzyme would be our target one) and avoids undesired side reactions that may be catalyzed by contaminant enzymes. However, purification of proteins is a process that may complicate the implementation of a biocatalyst because it is time consuming, high in cost, and may have

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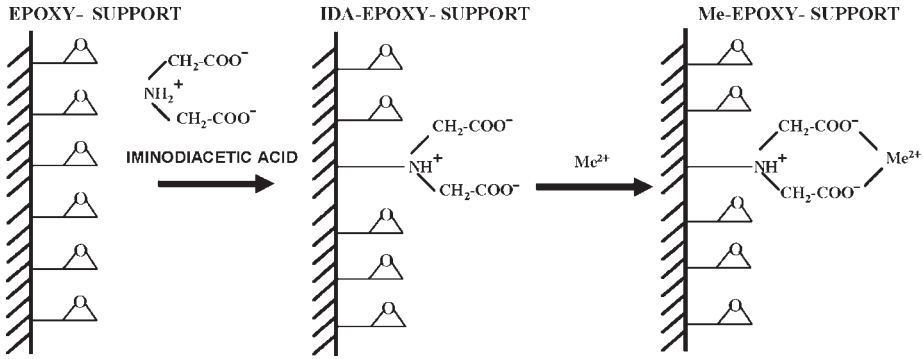

Fig. 1. Preparation of metal chelate–epoxy supports. Epoxy supports are incubated in the presence of iminodiacetic acid to introduce a few IDA groups in the support. The IDA supports are incubated with metal salts to obtain metal chelate–epoxy supports. Remained epoxy groups are blocked incubated them in presence of mercaptoethanol.

only partial yield (even just by enzyme inactivation). One suitable solution would be to join the purification and immobilization steps by designing a highly selective immobilization process.

Epoxy supports have been described as very suitable for the immobilization and stabilization of proteins via multipoint covalent attachment ***(1–10)***.

The mechanism of enzyme immobilization in epoxy supports ***(11–14)*** provides new opportunities for coupling immobilization to purification. It has been noted that the previous adsorption of protein on the epoxy support is necessary to achieve a significant covalent immobilization of the proteins because of the extremely low reactivity of the epoxy supports with soluble proteins ***(11–14)***. Using this premise, the use of multifunctional epoxy supports to immobilize proteins has recently been reported ***(14)***. This second generation of epoxy supports has different moieties that are able to physically adsorb proteins via different structural features, as well as a dense layer of epoxy groups that are able to covalently react with the enzyme. One type of multifunctional support that may be easily produced is the metal chelate–epoxy supports (CES; *see* **Fig. 1**) ***(15)***.

These supports may combine the good properties of epoxy supports for enzyme immobilization–stabilization with the enormous possibilities of immobilized metal chelate affinity chromatography (IMAC chromatography) to purify poly-His-tagged proteins (*see* **Fig. 2**). In this case, desorption of the contaminant adsorbed proteins is not possible, so higher selectivity than conventional IMAC chromatography will be required.

To achieve this selectivity,we have taken into account that natural proteins and poly-His tagged protein become adsorbed on IMAC supports following different mechanisms. The first requires interaction between different groups on the protein surface with several metal-chelate groups (see **Fig. 3**) ***(5)***, whereas poly-His-tagged proteins may be adsorbed by the interaction of the His tag with just one residue in the support ***(5)*** (*see* **Fig. 3**). This has been achieved by using low-superficial density of metal chelate in the support and metals that yield low-adsorption strength (e.g., Co^{2+}; *see* **Fig. 3**).

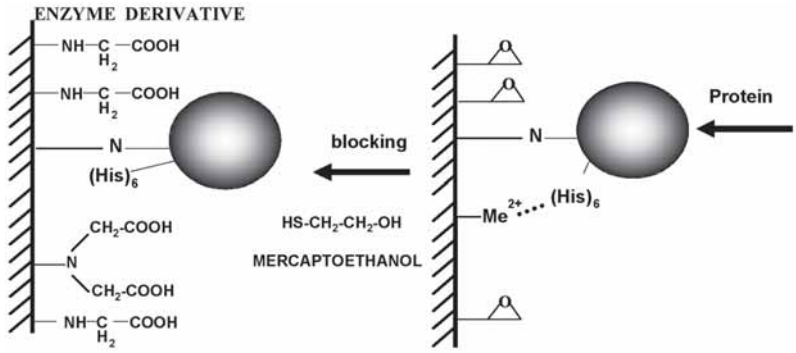

Fig. 2. Mechanism of the proteins immobilization on chelate supports. The IDA-Me^{+} supports are used to immobilize the proteins after they are incubated in the presence of mercaptoethanol to block the remaining epoxy groups and to release the Me^{2+} from the support.

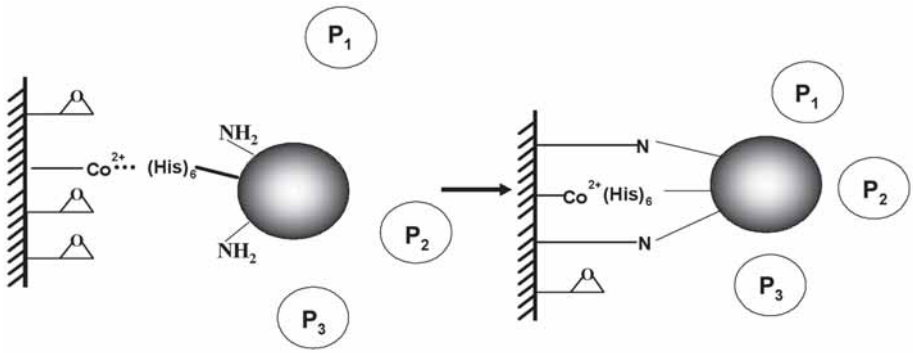

Fig. 3. Selective adsorption of poly-His-tagged proteins on lowly activated IDA-Co^{2+} epoxy supports.

Here, we present the preparation and use of this kind of heterofunctional support and its use in the one-step immobilization, purification, and stabilization of several poly-His-tagged proteins.

2. Materials

1. Epoxy Sepabeads® (FP-EP E902 P05) from Resindion SRL (Mitsubishi Chemical, Milan, Italy; *see* **Note 1**).
2. Eupergit® 250 from Degussa (40 µmols of epoxy groups per wet gram of support; *see* **Note 1**).
3. Iminodiacetic acid disodium salt monohydrate (IDA) was from Fluka (Buchs, Switzerland).
4. Support modification buffer: 0.1 *M* sodium borate, 2 *M* iminodiacetic acid, pH 8.5.

5. Metal containing solution: 0.05 *M* sodium phosphate buffer, pH 6.0, 1.0 *M* NaCl, and 5 mg/mL of $CoCl_2$.
6. Protein immobilization buffer: 0.05, pH 7.0, adjusted with 5 *M* NaOH.
7. Desorption buffer: 0.1 *M* imidazole in 5.0 m*M* sodium phosphate buffer, pH 7.0.
8. Imidazole was purchased from Merck (Darmstadt, Germany).
9. Incubation buffer: 0.1 *M* sodium phosphate buffer, adjusted to pH 8.5 or 10.0 with 5 *M* NaOH. In some instances, some additives could be added to keep enzyme activity, e.g., inhibitors. (*See* **Note 2**; not able to react with the epoxy supports.)
10. Blocking solution: 3 *M* glycine, pH 8.5.

3. Methods

3.1 Preparation of IDA–Epoxy Support

1. Incubate 10 g of Sepabeads or Eupergit in 18 mL of support modification buffer (*see* **Note 3**) under very gentle stirring for 2 h at 25°C.
2. Allow modification of around 5% of the epoxy-groups in the support.
3. Filter and wash the support with an excess of distilled water.

3.2 Preparation of IDA Epoxy–Metal–Chelate Support

1. Resuspended 10 g of IDA epoxy support (*see* **Subheading 3.1.**) in the metal containing solution for 2 h.
2. Allow chelation of all IDA groups in the support.
3. Filter and wash the support thoroughly with distilled water.
4. The metal content was analyzed by atomic adsorption spectroscopy.

3.3 Immobilization of Proteins on Epoxy Supports

1. The proteins were dissolved in the immobilization buffer (*see* **Subheading 2.**) and a sample was taken as reference (*see* **Note 2**).
2. The support was then added to the enzyme solution and submitted to a gently stirring (*see* **Note 3**).
3. Samples of supernatant and suspension were periodically taken. Supernatant was achieved by using pipet filter or by centrifugation of the suspension.
4. 2.5 mL Enzymatic suspension were taken and dried under vacuum filter.
5. The wet (but without inter-particle water) support was resuspended in 2.5 mL of desorption buffer. The suspension was left under stirring at 25°C for 30 min.
6. The activity or the protein concentration of the supernatant was checked.
7. If there are no activity releases then covalent attachment was considered.
8. The immobilized preparation was then washed five times with three volumes of incubation buffer and resuspended in three volumes of that buffer. Stirring is not necessary in this step.
9. The immobilized protein was left to interact with the support anywhere from 1 d to 1 wk. Activity of the immobilize preparations could be followed all along the incubation.

10. The immobilized preparations were vacuum dried and resuspended in three volumes of blocking solution under gentle stirring.
11. If the elimination of the metal was desired then the immobilized preparation was incubated with mercaptoethanol or EDTA.
12. Finally, the enzyme preparation was washed with distilled water and stored at 4°C.

3.4. One-Step Purification and Covalent Immobilization of Glutaryl Acylase

1. 5 g Eupergit 250 IDA-epoxy or Eupergit 250 was suspended in 15 mL of 0.05 *M* or 1 *M* sodium phosphate buffer, pH 8.5, respectively, at 25°C. Then, protein extract (glutary acylase [GA]) from *Escherichia coli*, (1 mg/mL) was added (*see* **Note 4**). Periodically, samples were withdrawn and the protein content of the supernatant and GA activity of supernatant and/or suspension was analyzed ***(17)***. Other samples of the support were incubated in presence of 0.1 *M* of imidazole, and both activity of supernatant and suspension were analyzed to study the covalent immobilization. This treatment was enough to desorb the entire enzyme (GA) from the fully modified chelate supports, without any remaining reactive epoxy group. After 24 h at pH 8.5, most of the enzyme was covalently attached to the bifunctional support (*see* **Fig. 4**). Interestingly, the immobilization protocol promoted only a slight decrease in enzyme activity (around 25%).
2. To determine the degree of purity of the immobilized enzyme, the enzyme derivatives were boiled in the presence of one volume of 2% sodium dodecyl sulfate (SDS). In this way, any molecule not covalently attached to the support was released into the medium. Then, SDS-polyacrylamide gel electrophoresis (PAGE), ***(18)*** analysis of the supernatant was performed and the gel was stained with Coomassie blue R-250 and analyzed by densitometry. **Figure 5** shows that most of the desorbed enzyme subunits belonged to GA, demonstrating the high selectivity of the Eupergit 250 IDA-epoxy immobilization protocol. However, when using other less specific adsorption protocols (e.g., conventional hydrophobic Eupergit 250 and an immobilization at high ionic strength), many different proteins can be detected in the supernatant after desorption treatment.
3. The activity of GA was evaluated in the hydrolysis of glutaryl 7-ACA in 0.05 *M* sodium phosphate buffer, pH 7.5, by titration of the glutaric acid released during the reaction by using 0.025 *M* NaOH.

3.5. Structural Stabilization of Glutaryl Acylase on Eupergit 250 IDA-Epoxy Supports Via Multipoint Covalent Attachment

1. Immobilized enzymes derivatives obtained in **Subheading 3.4.** were submitted to different incubation protocols after the adsorption process. Then, to analyze the degree of covalent attachment, they were boiled in the presence of SDS and the supernatants analyzed by SDS-PAGE. After enzyme immobilization at pH 8.5, it was possible to release most of the large β-subunits from the support (*see* **Fig. 6**), and there was therefore a lower proportion of small subunit (containing the poly-His-tag), which accounts for the percentage of enzyme physically adsorbed on the support at that time.

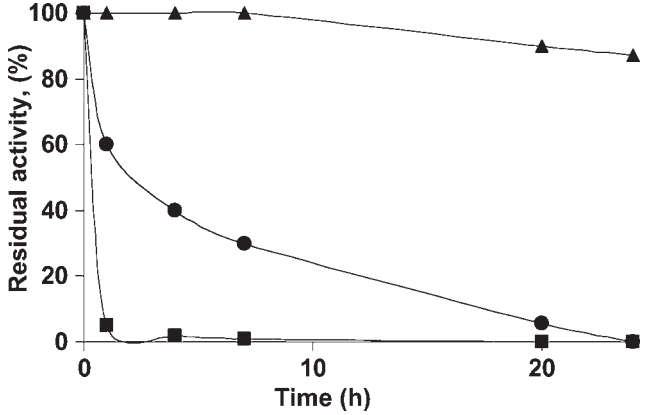

Fig. 4. Immobilization course of poly-His-tagged GA on Eupergit 250-IDA-Co support. Immobilizations were performed at pH 8.5 and 25°C in 50 m*M* sodium phosphate. (▲) Enzyme activity of the immobilization suspension; (●) enzyme activity on the supernatant of the immobilization suspension; (■) enzyme activity on the supernatant of the immobilization suspension after addition of 0.1 *M* imidazole (to release all enzyme molecules adsorbed only through the chelate groups of the support, but not covalently attached via epoxy groups to the support).

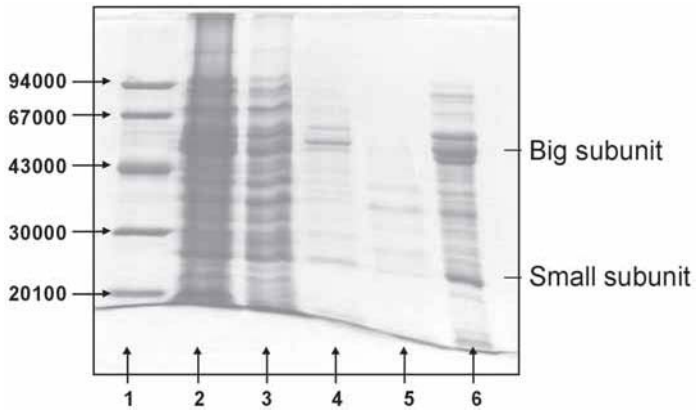

Fig. 5. Analysis by SDS-PAGE of the proteins (from a crude protein extract containing poly-His-tagged glutarayl acylase) immobilized on differently modified Eupergit 250 supports. Desorption of the proteins from the supports was carried out as previously described, in the case of the immobilized enzymes after the blocking of the epoxides with mercaptoethanol. *Lane 1*: molecular weight markers; *lane 2*: crude extracts from *E. coli*; *lane 3*: proteins in the supernatant after immobilization of the crude protein extracts on Eupergit 250-IDA-Co^{2+} at pH 7.0 and 15 m*M* sodium phosphate; *lane 4*: proteins released from the previous Eupergit 250-IDA-Co^{2+} derivatives by boiling it in the presence of SDS; *lane 5*: proteins in the supernatant after immobilization of crude protein extract on Eupergit 250 at pH 7.0 and 1 *M* sodium phosphate; *lane 6*: proteins released from the previous Eupergit 250 derivative by boiling in it the presence of SDS.

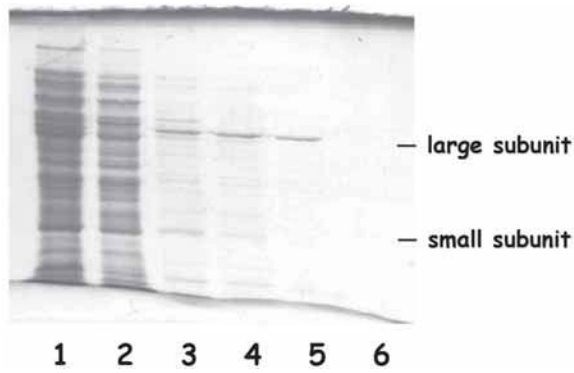

Fig. 6. Effect of pH in the multisubunit covalent immobilization of GA on Eupergit 250- IDA-Co supports. *Lane 1*, crude extract from *E. coli*; *lane 2*, supernatant after immobilization on Eupergit 250-IDA-Co supports; *lane 3*, supernatant after boiling in the presence of SDS of the derivatives incubated during 10 h at pH 8.5; *lane 4*, supernatant after boiling in the presence of SDS, the previous derivative that was further incubated during 1 h at pH 10.0; *lane 5*, supernatant after boiling in the presence of SDS, the derivative from *lane 3* after incubation during 8 h at pH 10.0; *lane 6*, supernatant after boiling in the presence of SDS the derivative from *lane 3* after incubation during 24 h at pH 10.0. The immobilized preparations were in all cases blocked with mercaptoethanol before the desorptions.

The incubation of the enzyme at pH 10.0 produced a rapid, progressive decrease in the amount of desorbed enzyme subunits. Thus, after 24 h of incubation, there was no detectable release of the two enzyme subunits after desorptions treatment. This result suggests that a multipoint covalent attachment between the enzyme and support was produced; that is, each enzyme molecule was attached by at least one bond per protein subunit to the support (see **Fig. 6,** *lane 6*).

2. The structural stabilization of GA was associated with an increase in enzyme stability. **Figure 7** shows that the immobilized derivative further incubated at pH 10.0 for 24 h (i.e., containing both subunits covalently attached to the support) was much more stable than the soluble enzyme but also presented multiphase inactivation, suggesting some heterogeneity in the degree of enzyme–support attachment. The average increase in the enzyme's half-life was around 100-fold when compared with the soluble enzyme, although a high percentage of immobilized enzyme presented greatly increased stabilization (>1000-fold more stable than soluble enzyme).

3.6. One-Step Purification and Covalent Immobilization of a Thermophilic Poly-His-Tagged β-Galactosidase From Thermus *sp. Strain T2*

1. 1 g Sepabeads FP-EP or Sepabeads FP-EP IDA-epoxy was incubated with 10 mL of crude protein extract containing poly-His-tagged β-galactosidase dissolved in 50 m*M* sodium phosphate pH 7.0 (*see* **Note 5**). Aliquots were withdrawn at regular time intervals, and the protein content of the supernatant and the activity of supernatant and/or suspension were analyzed ***(17)***. The immobilization was

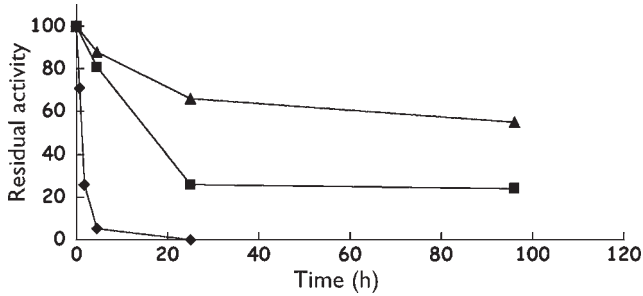

Fig. 7. Effect of the immobilization and further enzyme–support incubation of the derivative on the thermal stability of poly-His-tagged GA. Inactivation courses of different GA derivatives were performed at 45°C in 50 m*M* phosphate buffer, pH 7.5. (●) soluble enzyme; (■) derivative immobilized at pH 8.5; (▲) previous derivative further incubated at pH 10.0 during 24 h.

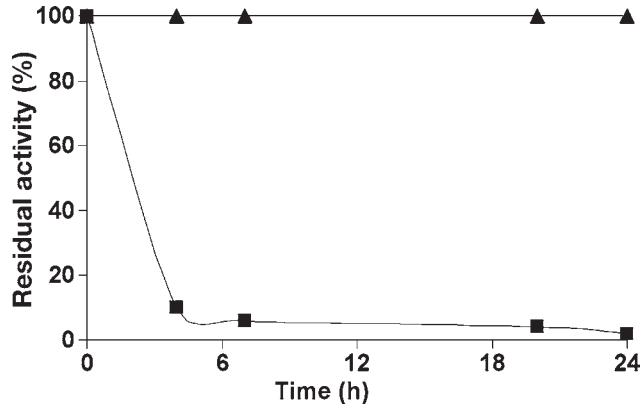

Fig. 8. Immobilization course of crude extract of poly-His-tagged β-galactosidase from *Thermus* spp. Strain T2, on IDA-Co-Epoxy–chelate support. (▲) enzyme activity of the immobilization suspension; (■) enzyme activity on the supernatant of the immobilization suspension.

stopped when covalent immobilization of the proteins was verified as described in **Subheading 3.3.**, **steps 5–7**. The use of Sepabeads FP-EP IDA-epoxy allowed the preparation of an immobilized enzyme derivative almost fully loaded with poly-His-tagged protein using as starting material a crude protein extract with only 3 to 5% of enzyme content. Moreover, the residual activity of the enzyme derivative was virtually 100 % (*see* **Fig. 8**).

2. To confirm selectivity of immobilization, the enzyme derivatives were boiled in the presence of one volume of 2% SDS, and the supernatants analyzed by SDS-PAGE. The immobilization on the bifunctional support seemed to be much more

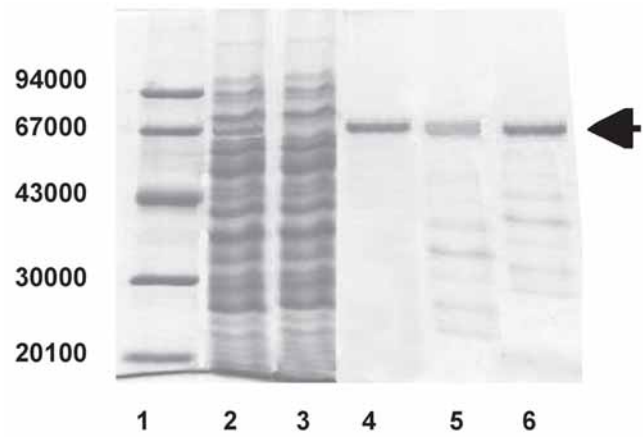

Fig. 9. Analysis by SDS-PAGE of the selectivity of the immobilization of poly-His-tagged β-galactosidase on different Sepabeads supports. *Lane 1*, molecular weight markers; *lane 2*, crude preparation of β-galactosidase*; lane 3*, proteins that are not immobilized on IDA-Co-Sepabeads; *lane 4*, proteins that are desorbed from glycine-blocked IDA-Co-Sepabeads derivatives; *lane 5*, proteins that are not immobilized on standard Sepabeads; *lane 6*, proteins that are desorbed from glycine-blocked standard Sepabeads derivatives. The immobilized preparations were in all cases blocked with mercaptoethanol before the desorptions.

selective than the immobilization on the conventional one. **Figure 9** shows that although many protein subunits could be detected using the standard support, only a band of 67 kDa (which corresponds to the molecular weight of the monomer of Htag-BgaA) could be detected in the bifunctional support.

3. Activity standard assay was performed with *o*-nitrophenyl- β-D-galactopyranoside (ONPG) at 25°C. ONPG was dissolved in Novo buffer, pH 6.5, and used at a final concentration of 13.3 m*M*.
4. One unit of β-galactosidase activity is defined as the amount of enzyme that produces 1 μmol of *o*-nitrophenol per minute under the conditions described.

3.7. Structural Stabilization of Poly-His-Tagged β-Galactosidase From Thermus spp. *Strain T2 on Sepabeads FP-EP IDA-Epoxy Via Multipoint Covalent Attachment*

1. Immobilized enzymes derivative prepared on Sepabeads FP-EP IDA-epoxy were further incubated after covalent immobilization at pH 10.0 for 24 h. This was carried out to favored multipoint covalent attachment (*see* **Subheading 3.5.**). **Figure 10** shows that this derivative of Htag-BgaA was more stable than the soluble enzyme and even more stable than that prepared using the conventional epoxy supports. Moreover, this immobilized derivative retained almost 100% of the initial activity after several weeks of incubation at 50°C (temperature employed normally at industrial pasteurization processes; *see* **Fig. 11**).

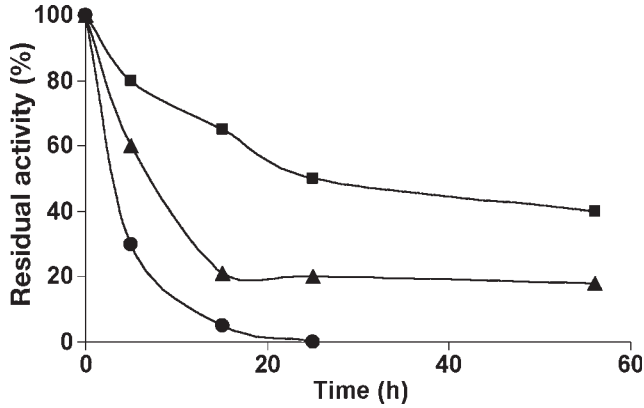

Fig. 10. Inactivation of different derivatives of β-galactosidase from *Thermus* sp. Strain T2. (●) Soluble enzyme; (▲) enzyme immobilized on standard epoxy support; (■) enzyme immobilized on IDA-CO-epoxy supports. Inactivation was carried out at pH 6.5 and 80°C.

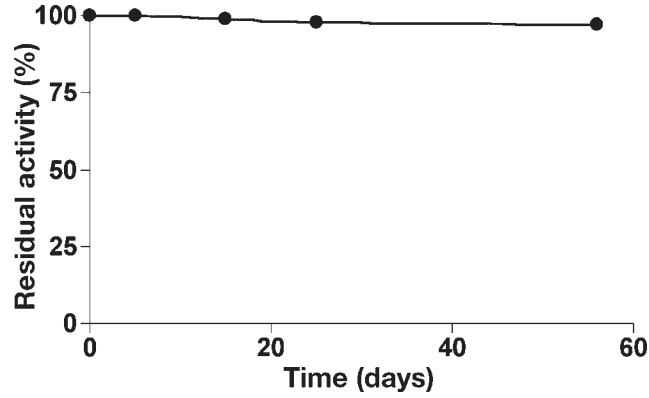

Fig. 11. Effect of the incubation at 50°C and pH 6.5 of the enzyme immobilized on IDA-CO-epoxy supports.

4. Notes

1. The epoxy supports (Sepabeads and Eupergit 250) must be stored dried at –20°C for the epoxy stability.
2. During covalent immobilization process, some enzymes may be inactivated at alkaline pH. In these cases, some inhibitors may be added to the immobilization/incubation buffer to protect the enzyme.
3. Stirring should be perfomed gently to prevent support breakage. This may promote some fines where diffusion problems are decreased and activity may apparently increase.

4. The poly-His-tagged GA extract was produced in *E. coli* as described previously ***(19)***.
5. Crude extract of poly-His-tagged β -galactosidase cloned and overexpressed in *E. coli* (MC1116) (Htag-BgaA) was produced as previously described ***(20)***. Only fresh enzyme preparations could be used to obtain immobilized enzyme derivatives with maximum loading. That results from the tendency of Htag-BgaA from *Thermus* strain T2 to self-associate in solution, and therefore to form large oligomeric species that obstruct the pores of the matrix ***(21)***.
6. The Novo buffer is designed to reproduce the composition of milk (2.7 m*M* sodium citrate, 7.91 citric acid, 2.99 m*M* potassium biphosphate, 10.84 m*M* potassium phosphate, 19.43 m*M* potassium hydroxide, 4.08 m*M* magnesium chloride, 5.1 m*M* calcium chloride, and 3.33 m*M* sodium carbonate) ***(22)***.

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12

Stabilization of Multimeric Enzymes Via Immobilization and Further Cross-Linking With Aldehyde–Dextran

Cesar Mateo, Benevides C. C. Pessela, Manuel Fuentes, Rodrigo Torres, Lorena Betancor, Aurelio Hidalgo, Gloria Fernández-Lorente, Roberto Fernandez-Lafuente, and Jose M. Guisan

Summary

Subunit dissociation of multimeric proteins is one of the most important causes of inactivation of proteins having quarternary structure, making these proteins very unstable under diluted conditions. A sequential two-step protocol for the stabilization of this protein is proposed. A multisubunit covalent immobilization may be achieved by performing very long immobilization processes between multimeric enzymes and porous supports composed of large internal surfaces and covered by a very dense layer of reactive groups. Additional cross-linking with polyfunctional macromolecules promotes the complete cross-linking of the subunits to fully prevent enzyme dissociation. Full stabilization of multimeric structures has been physically shown because no subunits were desorbed from derivatives after boiling them in SDS. As a functional improvement, these immobilized preparations no longer depend on the enzyme.

Key Words: Solid-phase chemical modification; aldehyde dextran cross-link; stabilization of quaternary structures; multisubunit immobilization.

1. Introduction

Protein immobilization may be of use not only to the re-use or continuous use of industrial enzymes. Immobilization and subsequent postimmobilization techniques can be useful for greatly increasing the activity–stability properties of industrial enzymes ***(1,2)***. In this chapter, the application of these techniques for the development of a general strategy to stabilize the quaternary structure of multimeric enzymes is discussed.

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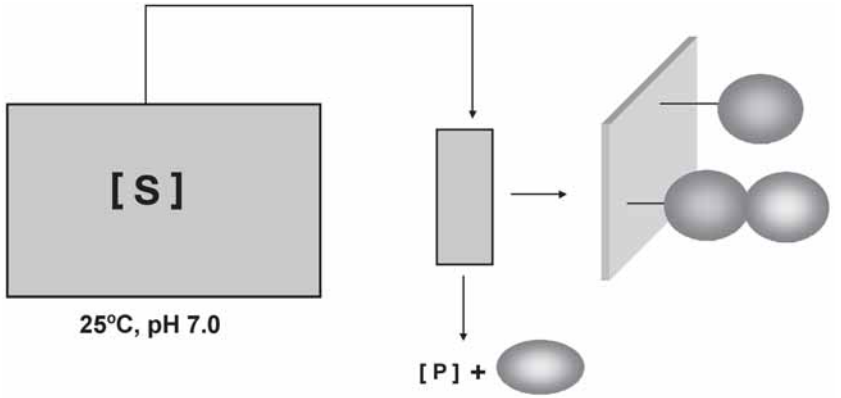

Fig. 1. Multimeric enzymes in industrial reactors.

Inactivation of multimeric enzymes may be strongly influenced by the dissociation of their subunits (*see* **Fig. 1**). Moreover, multipoint noncovalent interactions among monomers could stabilize the structure of each individual monomer correctly assembled in the multimer. On the contrary, conformational changes promoted by any denaturing agent (e.g., heat, pH, organic solvents) on the small fraction of dissociated monomers (not stabilized by these interactions) could be much more rapid ***(3)***. Dissociation of subunits may become even more relevant when working at the industrial scale. In general, we will try to use large volumes of reaction medium for many reaction cycles (*see* **Fig. 1**). The dissociation of a small fraction of subunits per reaction cycle could promote a very rapid deactivation of the biocatalyst. At first glance, it may be assumed that stabilization of the quaternary structure of multimeric enzymes may have very profitable effects on the industrial performance of these kind of enzymes.

1.1. Stabilization of Multimeric Enzymes Via Multisubunit Immobilization

A very intense chemical cross-linking between all the protein subunits should be one of the best approaches to stabilization of the quaternary structure as well as the stabilization of the correct enzyme assembly. In this way, an activated support may be considered as a very good cross-linking reagent. Thus, we propose the multisubunit attachment of multimeric enzymes to very rigid supports as a way to obtain the stabilization of the assembling shape and of the quaternary structure (*see* **Fig. 2**). Of course, the design of such multisubunit immobilization is not a trivial problem and it may require a careful selection of the immobilization systems. To intensify the support–enzyme interaction, it is necessary to control some parameters that affect the intensity of this interaction, and in general they will be similar to those controlling the multipoint covalent attachment ***(2)***. Among the main parameters, the activation degree of the support, the internal morphology of the support (e.g., plane surface vs thin fibers), and the immobilization conditions (e.g., pH, temperature, time, in general all the parameters that can improve the reactivity of the reactive groups of proteins and supports) have been described as parameters critical to stabilization of the multimeric enzymes.

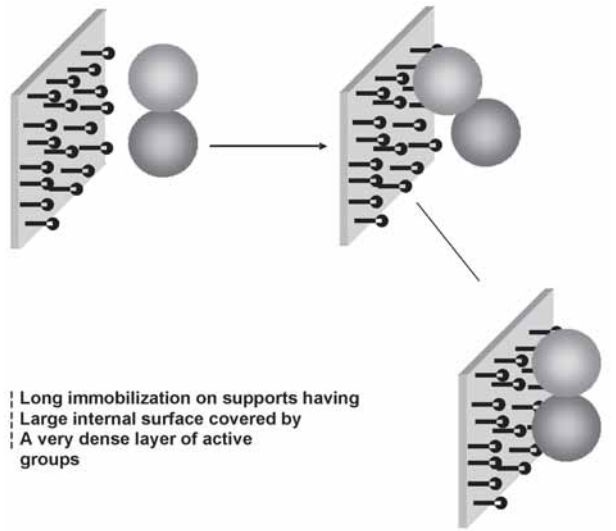

Fig. 2. Stabilization of multimeric enzymes via multisubunit immobilization.

1.2. Stabilization of the Quaternary Structure of Immobilized Multimeric Enzymes Via Multisubunit Cross-Linking With Dextran–Aldehyde

Immobilization of proteins under optimal conditions is not always enough to fully stabilize the quaternary structure of all multimeric enzymes. In some cases, the subunits are not in the same plane (e.g., tetrahedric enzymes), making it impossible to stabilize the enzymes solely via immobilization even in plane surfaces (*see* **Fig. 3**). In these cases the enzyme still can release subunits to the reaction medium and become easily deactivated.

To prevent this, cross-linking of subunits of the previously immobilized enzyme using multifunctional polymers such as polyaldehyde dextran has been described as a successful technology (*see* **Fig. 3**). Dextrans are polymers of *(1–6)* glucose, with some ramifications ***(1,2)*** commercially available in a broad range of molecular weights from different companies (Fluka, Sigma). Each monomer of glucose contains two points that can be easily oxidized by periodate to yield two aldehyde groups per molecule of glucose (*see* **Fig. 4**) ***(3)***. That enables the production of a polyaldehyde that can react with any primary amino group of the support or the enzyme surface. The reduction with sodium borohydride yields irreversible secondary amine bonds between the protein or the support, and the polymer. Moreover, this reduces the aldehyde groups that are presented in the dextran to a highly hydrophilic and inert polyhydroxyl.

This reagent may be very useful in intersubunit cross-linking because

- They have a high number of reactive groups able to react with primary amino group and quite frequent in the protein surface.
- Its large size may permit involvement in the cross-linking groups places in different protein subunits.

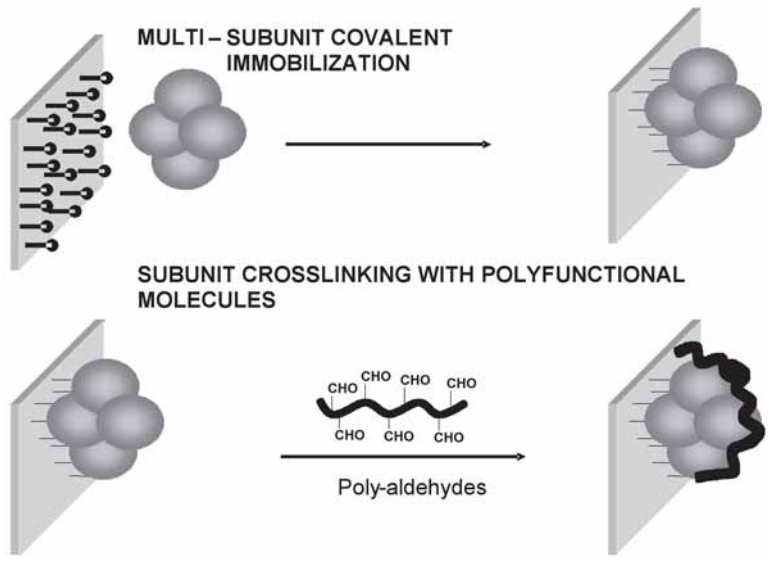

Fig. 3. An integrated approach to the stabilization of multimeric enzymes.

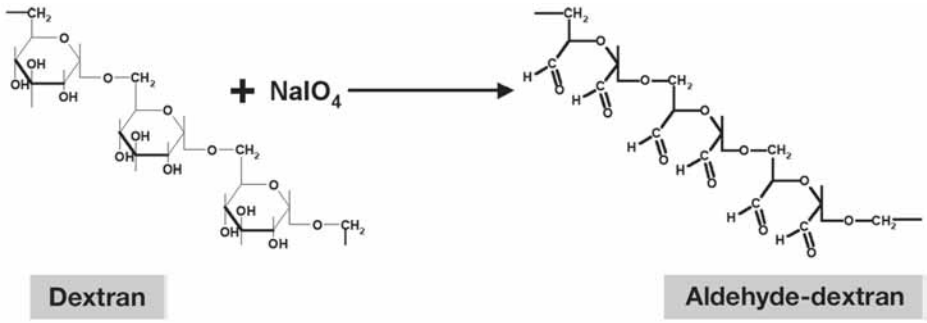

Fig. 4. Preparation dextran–aldehyde by sodium periodate oxidation.

- Its large size decrease the competition between one-point chemical modification and protein cross-linking.

2. Materials

1. Epoxy Sepabeads® (EP-2 or 3) from Resindion SRL (Milan, Italy; *see* **Note 1**.)
2. Eupergit® 250 from Degussa (*see* **Note 1**).
3. Commercial dextran (from *Leucosnostoc mesenteroides*) from Sigma or Fluka.
4. Glycidol (2,3-epoxy-propanol) sodium metaperiodate and sodium borohydride (Sigma, St. Louis, MO).
5. Dialysis membrane (MW cut: 12,000 Da) (Medicell Ltd., London, England).

6. Immobilization buffer: 1 *M* sodium phosphate at pH 8.0 adjusted with 5 *M* NaOH (*see* **Note 2**).
7. Incubation buffer: 100 m*M* sodium phosphate 9.0 adjusted at pH 9.0 with 5 *M* NaOH. Some additives could be added.
8. Blocking solution: 3 *M* glycine, pH 8.5.
9. Dextran to be oxidized was dissolved in distilled water.
10. Cross-linking buffer: 500 m*M* sodium phosphate, pH 7.0; adjusted with 5 *M* NaOH.
11. Reduction buffer: 100 m*M* sodium carbonate adjusted at pH 10.0 with 1 *M* NaOH at 4°C.
12. NOVO buffer: 2.7 m*M* sodium citrate, 7.91 m*M* citric acid, 1.03 m*M* potassium sulfate, 2.99 m*M* sodium dihydrogen phosphate, 10.84 m*M* disodium hydrogen phosphate, 19.43 m*M* potassium hydroxide, 4.08 m*M* magnesium chloride, 5.1 m*M* calcium chloride, 3.33 m*M* sodium hydrogen carbonate, and sodium hydroxide to achieve a pH value of 6.5.

3. Methods

3.1. Immobilization of Proteins on Epoxy-Supports

1. The proteins were dissolved in the immobilization buffer (*see* **Subheading 2.**) and a sample was taken as reference (*see* **Note 2**).
2. Then, the support was added to the enzyme solution and stirred gently (*see* **Note 3**).
3. Samples of supernatant and suspension were periodically taken. Supernatant was achieved by using pipet filter or by centrifugation of the suspension (*see* **Note 4**).
4. After 24 h, the immobilized preparation was washed five times with three volumes of incubation buffer, and then resuspended in three volumes of that buffer. Stirring is not necessary in this step.
5. The immobilized protein was left to interact with the support for different times (anywhere from 1 d to 1 wk). Activity of the immobilized preparations could be followed all along the incubation.
6. The immobilized preparation was vacuum dried and resuspended in three volumes of blocking solution and stirred gently for 24 h.
7. Finally, the enzyme preparation was washed with distilled water and stored at 4°C.

3.2. Preparation of Aldehyde–Dextran

1. Dissolve 1.25 g of dextran in 37.5 mL of distilled water (this gives an equivalent glucose concentration of 184.8 m*M*).
2. Add 3 g of 370 m*M* solid sodium periodate while stirring to dissolve the salt.
3. After 3 h, put the solution in a dialysis bag, and dialyze this against 5 L, changing the water every 3 h during the first 6 h and then changing every 8 h for a total of 22 h.
4. Use this solution after preparation to prevent any undesired reaction (*see* **Note 5**).

3.3. Modification of the Immobilized Enzyme With Aldehyde–Dextran

1. Wash 4 g of the immobilized enzyme five times with three volumes of cross-linking buffer.
2. Resuspend the immobilized enzyme in 40 mL of aldehyde–dextran solution.

3. Check enzyme activity along the process.
4. Periodically, take 4 mL samples of the suspension and add to 20 mL of reduction buffer.
5. Add 24 mg of sodium borohydride. Wash with distilled water after 30 min.

3.4. Physical Determination of the Stabilization of the Quaternary Structure of Multimeric Enzymes

The enzyme-support bonds are very stable secondary amino bonds, ethers, or thioether; therefore, no protein–subunit bond to the support should be released to the medium. Thus, to asses the stabilization of the quaternary structure, the following protocol was followed:

1. Incubate 1 g of immobilized proteins in 2 mL 4% (w/v) sodium dodecyl sulfate (SDS), in the presence of 10% mercaptoethanol.
2. Boil this suspension for 5 min to promote the release of any noncovalently immobilized protein.
3. Take samples of the supernatant and analyze using SDS-polyacrylamide gel electrophoresis (PAGE) techniques following standard protocols.

3.5. Functional Determination of the Stabilization of the Quaternary Structure of Multimeric Enzymes

From an applied point of view, the main effect of the stabilization of multimeric enzymes should be that the enzyme concentration has no effect on the enzyme stability. To asses that:

1. Add 10 mL of the desired inactivation buffer to 1 g of immobilized enzyme.
2. Stir that suspension and, using a pipet with a cut tip, remove 1 mL and add it to 10 mL of the same inactivation buffer.
3. Perform the inactivation experiment.

3.6. Stabilization of a Multimeric β-Galactosidase From Thermus sp. Strain T2 by Immobilization on Novel Heterofunctional Epoxy Supports Plus Aldehyde–Dextran Cross-Linking

The immobilized β-galactosidase derivatives (on Sepabeads–epoxy supports partially modified with boronate, iminodiacetic, etc) showed improved stabilities after favoring multipoint covalent attachment by long-term alkaline incubation. The enzyme immobilized on Sepabeads–epoxy-boronic was the most stable. This derivative had some subunits of the enzyme not covalently attached to the support (detected by SDS-PAGE). To determine the amount of protein subunits not covalently attached to the support in the β-galactosidase-boronic-epoxy-Sepabeads derivative (prepared using a crude preparation), the immobilized preparation was boiled in 2% SDS, and the proteins released to the supernatant were analyzed by SDS-PAGE. This analysis revealed that several subunits of multimeric proteins were not covalently attached to the support (*see* **Fig. 5**).

The quaternary structure of β-galactosidase from *Thermus* spp. was completely stabilized after incubation of the β-galactosidase–boronic-epoxy–Sepabeads derivative with aldehyde–dextrans under standard conditions ***(5,6)*** (*see* **Fig. 5**, *lane 3*).

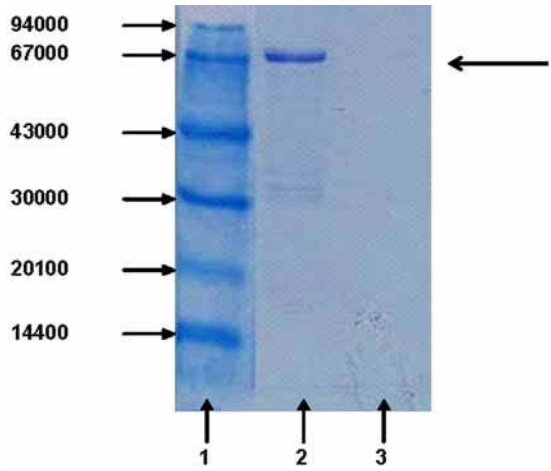

Fig. 5. 12% SDS-PAGE analysis of the β-galactosidase immobilized on boronic-epoxy–Sepabeads. *Lane 1*: molecular weight marker; *lane 2*, desorption of the protein subunits not covalently immobilized on Sepabeads–boronic-epoxy support; *lane 3*, desorption of proteins from Sepabeads–epoxy-boronate–β-galactosidase optimal derivative and cross-linked with polyaldehyde–dextran. Arrows indicate the position of the β-galactosidase. Experiments were carried out in triplicate. All other specifications were described in **Subheadings 2.** and **3**.

However, this treatment resulted in a decrease of almost 80% in the enzyme activity after 1 h of incubation (*see* **Fig. 6**). To reduce the activity decrease, the cross-linking process was carried out in the presence of different agents that could protect the active site of the enzyme, such as lactose (substrate) or galactose (competitive inhibitor). Interestingly, both compounds proved to have a protective effect, but the best result was achieved in the presence of 1 *M* lactose (*see* **Fig. 7**). Nevertheless, even in the presence of this protective reagent, the enzyme activity dropped by about 30% after this chemical modification. Therefore, other parameters such as dextran size, temperature, and incubation times were further optimized. Finally, it was observed that, using a dextran of 71,400 Da for 6 h at 4°C, 95% of the activity was recovered after chemical modification of the derivative. Moreover, this treatment succeeded in stabilizing the quaternary structure of all immobilized multimeric enzymes because no protein band, even using unpurified extracts (neither from the target enzyme nor from other multimeric enzymes), was detected when the cross-linked Sepabeads–boronic-epoxy derivative was boiled in SDS-PAGE (*see* **Fig. 5**, *lane 3*). This derivative retained a very high thermostability (*see* **Fig. 8**) and did not release any contaminant protein during the reaction, satisfying the requirements for their industrial use in food technology.

This is a problem if the biocatalysts were to be used in food technology. The optimization of the cross-linking with aldehyde–dextran permitted the full stabilization of the quaternary structure of the enzyme.

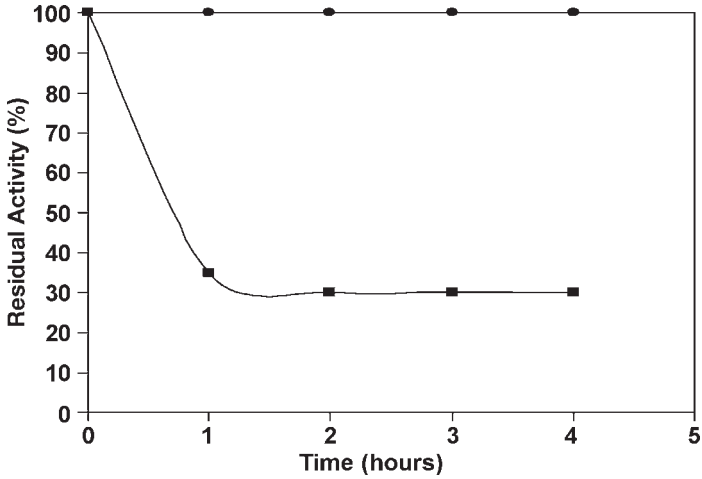

Fig. 6. Effect of cross-linking with dextran–aldehyde on the activity of β-galactosidase immobilized onto Sepabeads–epoxy-boronate support. Time course of inactivation of β-galactosidase–Sepabeads–epoxy-boronate derivative by incubation with aldehyde–dextran. (●) Sepabeads–epoxy-boronate–β-galactosidase derivative. (■) Sepabeads–boronic-epoxy–β-galactosidase derivative incubated with aldehyde–dextran as described in **Subheading 3.3.**

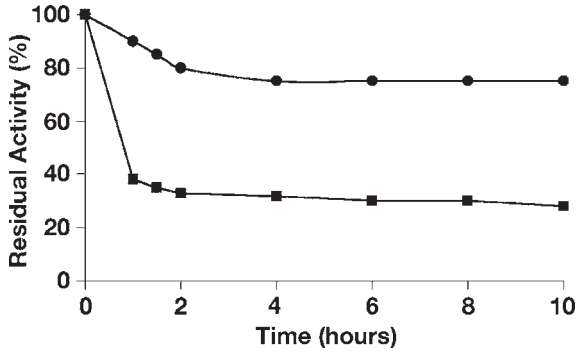

Fig. 7. Effect of the presence of lactose on the inactivation of Sepabeads–boronic-epoxy–β-galactosidase derivative during the incubation with aldehyde–dextran. The assay was carried out in Novo buffer, pH 6.5) at 25°C. Values were reproduced in two separate experiments. All other conditions are detailed in **Subheadings 2.** and **3**. (●) Sepabeads–boronic-epoxy–β-galactosidase derivative cross-linked with aldehyde–dextran incubated in the presence of 1 *M* lactose. (■) Sepabeads–boronic-epoxy–β-galactosidase derivative cross-linked with aldehyde–dextran.

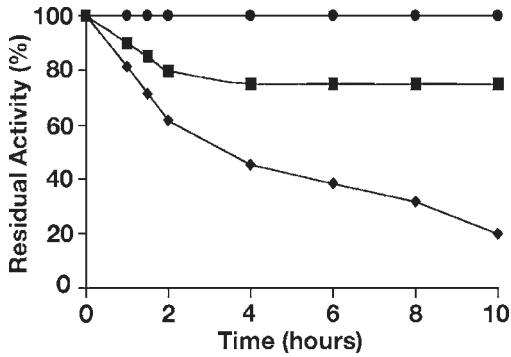

Fig. 8. Functional inactivation of optimal cross-linked derivative. Thermal stability course of Sepabeads–boronic-epoxy–β-galactosidase derivative at 80°C. (●) cross-linked with aldehyde–dextran Sepabeads–boronic-epoxy–β-galactosidase; (■) Sepabeads–boronic-epoxy–β-galactosidase derivative cross-linked with aldehyde–dextran and protected with 1 *M* lactose; and (◆) soluble β-galactosidase.

3.7. Stabilization of the Quaternary Structure of α-Amino Acid Ester Hydrolase From Acetobacter turbidans *Via Immobilization and Postimmobilization Techniques*

3.7.1. Functional Stability

The stability of this tetrameric enzyme in soluble form is quite low and decreased with decreasing enzyme concentration (*see* **Fig. 9**), suggesting that some dissociative process may be the key step of the enzyme inactivation. The immobilization of the enzyme onto supports with low-activation degree has a negligible effect on its stability, even when using longer immobilization times (e.g., 24 h). When using these low-activated supports, the dependence of the immobilized enzyme stability on the protein concentration was quite similar to that of the soluble counterpart (*see* **Fig. 9**).

On the contrary, when using highly activated supports, the thermal stability of the enzyme was significantly improved. Such stabilization increased when the enzyme–support reaction time increased, reaching its maximum after 24 h of contacting the enzyme with the support. In this derivative, the dependence of the enzyme stability on the protein concentration becomes smaller than in the soluble enzyme, but it is still quite significant.

The modification of such immobilized and partially stabilized derivative with aldehyde–dextran promoted a further stabilization of the enzyme (*see* **Fig. 9**). Moreover, the stability of the enzyme derivative did not depend on the concentration of protein. This derivative was much more stable than both the soluble enzyme and the enzyme that was immobilized onto low-activated supports (*see* **Fig. 9**). These results suggest that dissociation of the enzyme subunits may be responsible for the low stability of the soluble enzyme.

On the other hand, **Fig. 10** shows that the stability of the soluble enzyme was extremely dependent on the phosphate concentration. The enzyme stability was

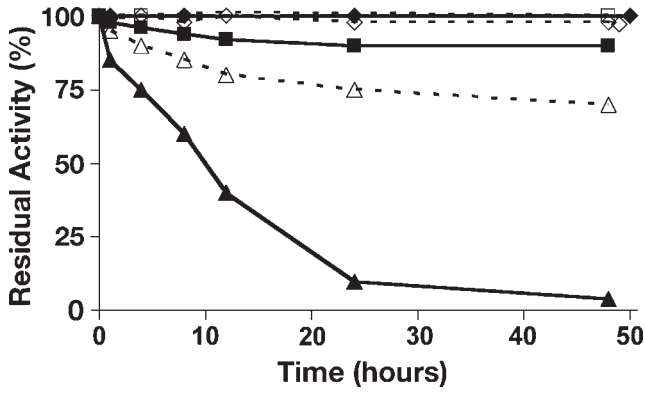

Fig. 9. Thermal inactivation of different preparations of α-amino acid ester hydrolase from *A. turbidans*. Inactivation was carried out in 25 m*M* phosphate at 25°C, pH 6.5, using purified enzyme. Solid lines, solid symbols: concentration of protein was 1 µg/mL, Dashed lines, empty symbols: concentration of protein was 10 µg/mL. (▲) Soluble enzyme and immobilized in low-activated glutaraldehyde supports. (■) Enzyme immobilized on highly activated glutaraldehdye supports. (◆) Previous derivative further modified with polyaldehyde–dextran.

very low in the absence of phosphate, and the effect of the protein concentration was even more relevant than in 100 m*M* phosphate. However, the immobilized and chemically cross-linked derivative remained fully active even in the absence of phosphate ions for very long incubation times (*see* **Fig. 10**). These results suggest that the phosphate ions may be somehow related to the maintenance of the quaternary structure of the enzyme.

The immobilization and modification of the enzyme promoted only a slight decrease in its activity (~15–20%) while increasing the operational stability of the diluted enzyme by several orders of magnitude.

3.7.2. Structural Stabilization

Figure 11 shows the SDS-PAGE gel obtained after boiling the different derivatives in the presence of SDS, offering in all cases similar amounts of protein. *Lane 2* shows the purified enzyme with a main band corresponding to a MW of around 70 KD (corresponding to the MW of each monomer of this enzyme). After being subjected to the same treatment, the derivative prepared in low-activated glutaraldehyde support still produced that band, with an intensity of about 70% of that observed for the purified enzyme. This result indicated that only one of the four protein subunits has been covalently attached to the support and may explain why the stability of this derivative was almost identical to that of the soluble enzyme (the possibilities of subunit dissociation would be almost identical).

When analyzing the derivative prepared using highly activated agarose-glutaraldehyde, after 24 h of enzyme support multi-interaction, some protein could still be released from the support by the desorption treatment, but the relative intensity of the band was now between 30 and 40% (regarding the pure enzyme), suggesting

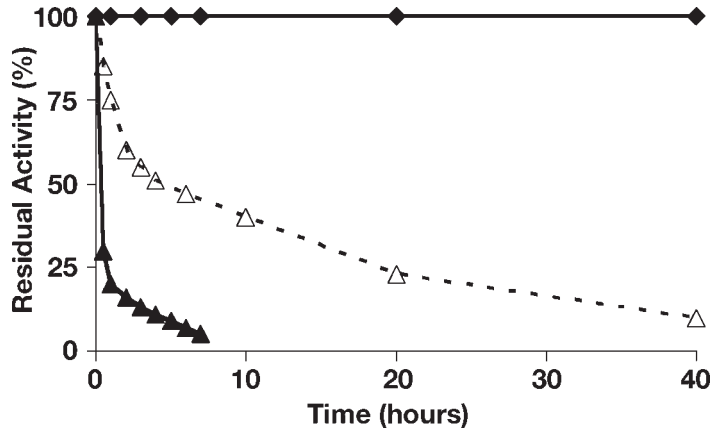

Fig. 10. Effect of phosphate concentration on the stability of α-amino acid ester hydrolase from *A. turbidans*. Inactivations were performed at 25°C, pH 6.5, with a fixed concentration of protein (5 µg/mL). (◆) Stabilized enzyme derivative at 0 or 100 m*M* of sodium phosphate. (△) Soluble enzyme in 100 m*M* phosphate, (▲) Soluble enzyme in 100 m*M* NaCl or water.

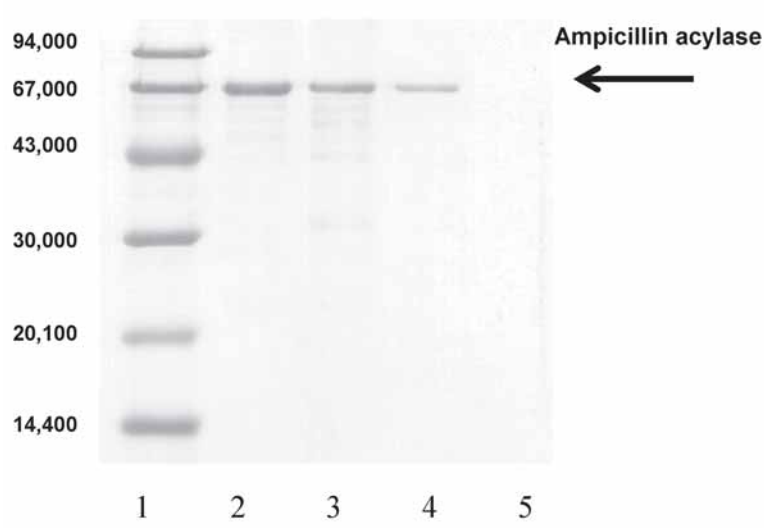

Fig. 11. Desorption of noncovalently immobilized protein subunits from the support in the prepared derivatives. Desorption was carried out by boiling the derivatives in the presence of SDS. *Lane 1*, crude extract; *lane 2*, fraction of purified enzyme; *lane 3*, enzyme immobilized on agarose–glutaraldehyde poorly activated (1 h of immobilization); *lane 4*, **e**nzyme immobilized on agarose–glutaraldehyde highly activated (24 h of immobilization); *lane 5*, previous derivative modified with polyaldehyde–dextrans.

that at least two to three of the four subunits of the enzyme had been covalently attached to the support. However, some of the subunits were not yet covalently attached to the support and could dissociate from the whole protein. These results may explain the significant increase in the enzyme stability and the significant decrease of the dependence of the enzyme stability on the enzyme concentration found when studying this derivative.

The derivative that had been chemically cross-linked with aldehyde–dextran, after boiling in SDS, did not release any detectable protein to the supernatant. Therefore, this treatment seems to be able to fully stabilize the quaternary structure of this complex tetrameric enzyme (*see* **Fig. 11**, *lane 5*). This explains the nondependence of the stability of the enzyme on the enzyme concentrations when analyzing this derivative, and also explains the high stability of such enzyme derivative in the absence of any phosphate ions.

Thus, this simple two-step treatment is able to fully stabilize the quaternary structure of the enzyme.

4. Notes

1. The support should be selected as a function of the protein size: in the case of agarose, the larger the percentage of agarose, the lower the pore diameter; in the case of Sepabeads, the higher the number, the higher pore size. Other activation of the support (e.g., glioxyl, glutaraldehyde) may be utilized to fulfil this multisubunit immobilization.
2. The immobilization of multimeric enzymes may require the use of high-protein concentrations and the addition of certain compounds to keep the multimeric structure, even just during the immobilization time.
3. Magnetic stirring may act as a mill stone, when any kind of support is used. Other types of stirring devices should be used (e.g., shaker, orbital stirring, mechanical stirring)
4. Use cut pipet tips to measure the suspension.
5. Aldehyde dextran should never be exposed at a pH over 8.0 except during reduction. If the preparation takes a brown color, it should be discarded.

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13

Purification, Immobilization, Hyperactivation, and Stabilization of Lipases by Selective Adsorption on Hydrophobic Supports

Jose M. Palomo, Gloria Fernández-Lorente, Cesar Mateo, Rosa L. Segura, Claudia Ortiz, Roberto Fernandez-Lafuente, and Jose M. Guisan

Summary

Immobilization of lipases on hydrophobic supports at low ionic strength permits one-step purification, immobilization, hyperactivation, and stabilization of most lipases. This selective adsorption occurs because the hydrophobic surface of the supports is able to promote the interfacial activation of the lipases, yielding enzyme preparations having the open form of the lipases very strongly adsorbed on these hydrophobic supports. At low ionic strength, only proteins having large hydrophobic pockets may become adsorbed on the hydrophobic support, and the only soluble proteins are lipases, which in closed form are fairly hydrophilic, but in open form expose a very hydrophobic pocket. The resulting biocatalysts are therefore hyperactivated, at least with hydrophobic and small substrates (because all the enzyme molecules have the open form). Moreover, the stabilization of the open form of the lipases permits very highly stabilized enzyme preparations.

Key Words: Interfacial activation; hydrophobic supports; stabilization of the open form of lipases; selective adsorption; lipase stabilization.

1. Introduction

Lipases (E.C. 3.1.1.3) have as natural function the hydrolysis of triglycerides. Nevertheless, they may be used in vitro to catalyze many different reactions, in many instances quite far from the natural ones (e.g., regarding conditions, substrates). Thus, lipases are utilized in different industrial applications, such as in the production of modified oils ***(1,2)***, cosmetics ***(3,4)***, or—the most important one in the last years—production of many different intermediates for organic synthesis (e.g., resolution of racemic mixtures), because they combine broad substrate specificity with a high enantio- and regioselectivity ***(5–10)***.

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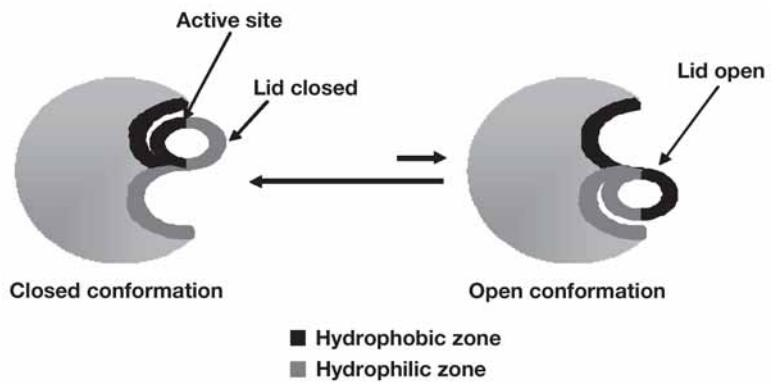

Fig. 1. Interfacial adsorption of lipases.

However, these enzymes present a very complex catalytic mechanism. In homogeneous aqueous solutions, the lipase is mainly in a closed and inactive conformation where the active site is completely isolated from the reaction medium by an oligopeptide chain (called flap or lid), blocking the entry of substrates to the active site. This polypeptide chain presents several hydrophobic amino acid residues in its internal face, interacting with hydrophobic zones around the active site. This conformation may exist in a partial equilibrium with an open and active conformation, where the lid is displaced, stabilized by ionic interactions or hydrogen bonds with a specific part in the lipase surface allowing the access of the substrate to the active site ***(11,12)***.

However, upon exposure to a hydrophobic interface such as a lipid droplet, the lipase can only interact (when it is in the open conformation), via the hydrophobic pocket formed by the internal face of the lid and the surroundings of the active site, consequently shifting the equilibrium towards the open form—the so called interfacial activation ***(13,14)*** (*see* **Fig. 1**).

This mechanism of action promotes some problems when industrial immobilized lipases are prepared. Inside a porous structure, lipases molecules become inaccessible to any kind of external interfaces, therefore there is no possibility of enzyme interfacial adsorption in aqueous solutions. In fact, conventionally immobilized lipase preparations are usually utilized primarily in anhydrous media, where the lipase may become activated by the direct interaction with the organic solvent phase ***(15)***.

However, many interesting reactions catalyzed by lipases may be advantageously carried out in aqueous systems (e.g., hydrolytic resolutions of racemic mixtures) ***(8–10)***. The interfacial adsorption of lipases on hydrophobic supports has been proposed as a simple method for preparing immobilized lipase preparations useful in any media ***(16,17)***. The hypothesis behind this immobilization strategy is to take advantage of the complex mechanism of lipases (an apparent problem) as a tool that permits the immobilization of lipases via an “affinity-like” strategy ***(16,17)***. Using a hydrophobic support (that somehow resembles the sur-

face of the drops of the natural substrates) and very low ionic strength, lipases become selectively immobilized on these supports. The adsorption involves the hydrophobic areas surrounding the active center and located in the internal face of the flat (no other water-soluble proteins are adsorbed on the support under these mild conditions) ***(16–18)***. These adsorbed lipases are able to access the active center; in fact, immobilized enzymes usually exhibit significantly enhanced enzyme activity (by the "interfacial adsorption mechanism"). The result is an immobilized lipase where the open conformation has been "fixed" and does not depend on the presence of external hydrophobic interfaces.

2. Materials

1. Lipases from *Candida antarctica* B, *Rhizomucor miehei*, *Humicola lanuginosa* (Novo Nordisk, Denmark)
2. Lipases from *Rhizopus niveus*, *Mucor javanicus*, and *Pseudomonas fluorescens* (Amano Co., Nagoya, Japon).
3. Lipase from *Candida rugosa*, (Sigma, St. Louis, MO).
4. *p*-Nitrophenyl propionate (*p*-NPP), (Sigma).
5. Triton X-100 (Sigma).
6. Ethyl butyrate (Sigma).
7. Buffer for immobilization: 25 m*M* sodium phosphate buffer adjusted at pH 6.0, pH 7.0.
8. Assay buffer: 25 m*M* phosphate, pH 7.0.
9. Butyl-, phenyl-, and octyl-Sepharose 4BCL support (Pharmacia Biotech, Uppsala, Sweden).
10. Octadecyl-Sepabeads® resin (Resindion, SRL; Milan, Italy).
11. Shimadzu UV (UV250PC).
12. pHstat (Mettler Toledo, Madrid, Spain).

3. Methods

3.1. Activity Assay of Lipases

1. A 50-m*M* stock solution in acetonitrile of the substrate (*p*NPP) was prepared.
2. 2.5 mL Phosphate buffer and 20 µL of substrate stock solution were added to a spectrophotometric cell and the mixture was preincubated at 25°C for 10 min.
3. Esterasic lipase activity was measured using an ultraviolet spectrophotometer by measuring the increase in the absorbance at 348 nm produced by the release of *p*-nitrophenol in the hydrolysis of *p*NPP prepared as described above at pH 7.0 and 25°C.
4. 0.05 mL Lipase solution or suspension (1 U/mL) was added to 2.5 mL of *p*NPP solution (*see* **Note 1**).

3.2. Immobilization of Lipases on Hydrophobic Supports

1. Wash 10 g of support 10 to 15 times with three volumes of distilled water.
2. Mix 1 mg/mL of the enzyme solution and 10 g of swelling support in 5 to 10 m*M* sodium phosphate immobilization buffer, pH 7.0. Stir gently for 3 h at 25°C (*see* **Note 2**).

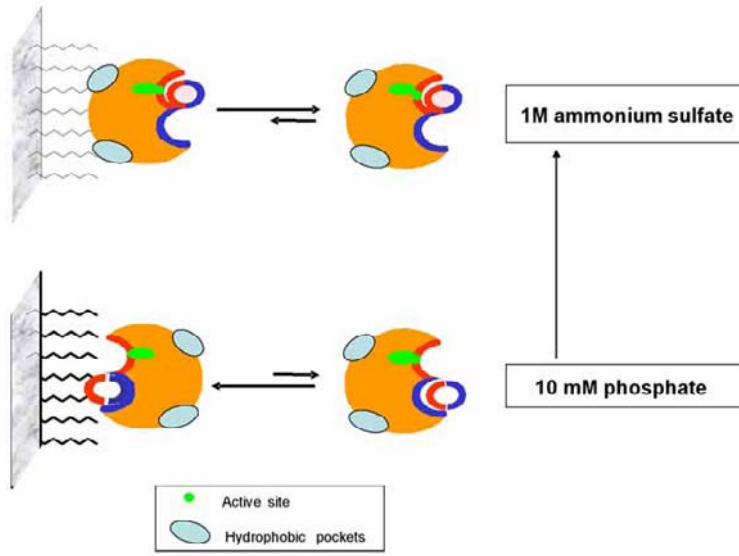

Fig. 2. Adsorption of proteins/lipases on hydrophobic supports.

3.3. SDS-PAGE of the Immobilized Enzymes

1. Incubate 1 g of immobilized enzyme in 2 mL 4% (w/v) sodium dodecyl sulfate (SDS) in the presence of mercaptoethanol and boil suspension for 5 min. This promotes the release of any adsorbed lipase.
2. Take samples of the supernatant and analyze following standard protocols (*see* **Fig. 2**).

3.4. Effect of the Adsorption on Hydrophobic Supports in the Stability of Lipases

1. Resuspend 1 g of the desired lipase immobilized preparation in 25 m*M* sodium phosphate buffer, pH 7.0, in the desired temperature.
2. Periodically withdraw samples using tip-cut pipets and measure the activity as described in **Subheading 3.1**.

3.5. Stability of Lipase-Hydrophobic Preparations in the Presence of Organic Cosolvents

1. Different cosolvents at different concentrations are prepared, using Tris-HCl as a buffer in order to prevent pH changes (*see* **Note 3**).
2. Wash the enzyme preparation 10 times with three volumes of the desired organic solvent/buffer mixture at 4°C.
3. Resuspend 1 g of the desired lipase immobilized preparation in the previous solution at the desired temperature and measure the activity immediately (*see* **Note 4**) as described in **Subheading 3.1**.

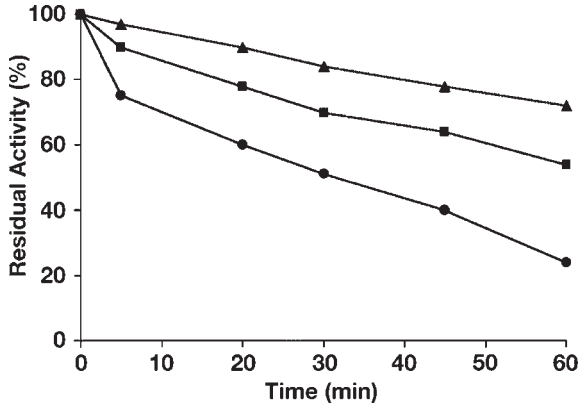

Fig. 3. Effect of ionic strength in the adsorption of lipase from *R. niveus* to octyl-agarose. 1 mL Octyl-agarose was added to 200 mL of lipase suspension (0.1 mg of protein/mL) at 48°C, pH 7.0), in 10 m*M* phosphate and different concentrations of ammonium sulfate: without ammonium sulfate (●), 0.1 *M* ammonium sulfate (■), 1 *M* ammonium sulfate (▲). Activity of supernatant was measured in all cases.

4. Periodically, withdraw samples using tip-cut pipets and measure the activity as described in **Subheading 3.1**.

3.6. Desorption of the Adsorbed Lipase From the Hydrophobic Support

1. After inactivation of the biocatalyst the lipases may be desorbed through use of detergents or organic buffer/organic solvents if it is required at higher temperatures.

3.7. Effect of the Ionic Strength on the Lipase Adsorption on Hydrophobic Support

Conventional hydrophobic adsorption of proteins is stronger and more rapid when increasing the ionic strength (*see* **Fig. 2**). However, adsorption of lipases on octyl-agarose support was slowed down when the ionic strength increased (*see* **Fig. 3**) ***(16)***. Maximum immobilization rates were obtained at only 5 to 10 m*M* of sodium phosphate buffer.

3.8. Selectivity of the Immobilization on Hydrophobic Supports

The full activity of lipases from very different sources *(Candida rugos*a, *C.antarctica* B, *Humicola lanuginosa*, *Pseudomonas fluorescens*, *Rhizopus niveus*, *Rhizomucor miehei*, *Thermus aquaticus*, *Bacillus thermocatenulatus*) was quickly adsorbed—less than 10 min in relation to a 1-mL gel and 10 mL of lipase extract—at low ionic strength (5–10 m*M* sodium phosphate). Furthermore, the other proteins contained in the crude extracts remained in the solution ***(16,17,19)*.**

In this way, an unique band corresponding to the exact mass of the lipase was observed in the SDS-PAGE for different enzymes adsorbed on the octyl-agarose support (*see* **Fig. 4**). Therefore, this easy and quick immobilization methodology can be use to purify lipases.

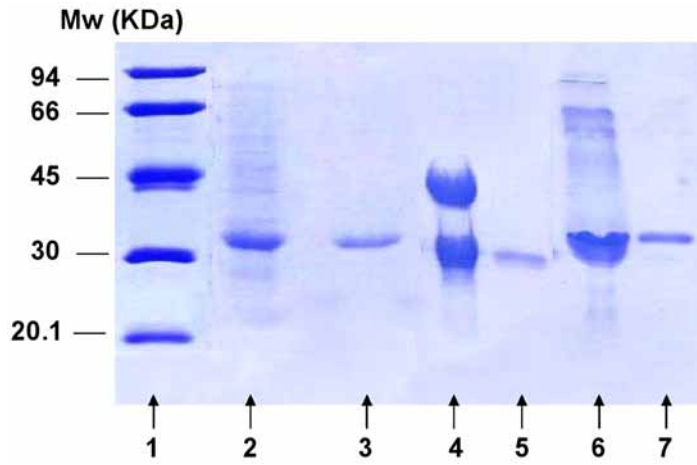

Fig. 4. Analysis of the adsorption of different lipase extracts on octyl-agarose by SDS–PAGE. *Lane 1*, molecular weight markers; *lane 2*, commercial *P. fluorescens* preparation; *lane 5*, *P.fluorescens* lipase adsorbed on octyl-agarose support; *lane 4*, commercial *H. lanuginosa* preparation; *lane 3*, *H. lanuginosa* lipase adsorbed on octyl-agarose support; *lane 6*, commercial *C. antartica B* preparation; *lane 7*, *C. antarctica* B lipase adsorbed on octyl-agarose support.

3.9. Hyperactivation of Lipases Resulting From the Interfacial Activation Against the Hydrophobic Support

The immobilization of lipases on hydrophobic supports (octyl-agarose or octadecyl-Sepabeads®) promoted a significant increase in the lipase activity (hyperactivation) against completely soluble substrates. **Figure 5** shows a typical adsorption immobilization experiment, in which suspension activity increases during immobilization.

3.10. Stabilization of Lipases by Interfacial Activation Against Hydrophobic Supports

Furthermore, these immobilized preparations were very stable biocatalysts, much more that the soluble one, in the thermal-inactivation (*see* **Fig. 6**). The enzyme adsorption is so strong that there is no desorption of the protein, even in 40% organic solvents such as dioxane. In fact, in the presence of organic cosolvents the enzyme also exhibited a high increment of stability (*see* **Fig. 7**)

3.11. Separation of Lipase Isozymes and Isoforms by Adsorption on Agarose With Different Hydrophobicity

Several lipase isoforms have been described in many organisms. These different isoforms could have different properties when catalyzing organic reactions. However, the separation of very similar enzyme structures may be extremely difficult using conventional chromatographic techniques. Thus, very small modifications on enzyme structure could promote important changes in the mechanism of

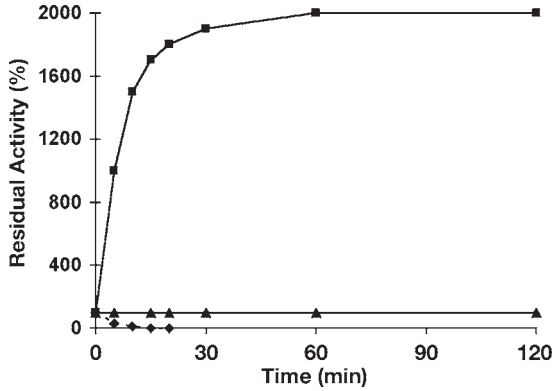

Fig. 5. Hyperactivation of lipase from *H. lanuginosa* during its adsorption on octyl-agarose. Supernatant (◆), immobilization suspension (■), blank suspension, with inert agarose (▲).

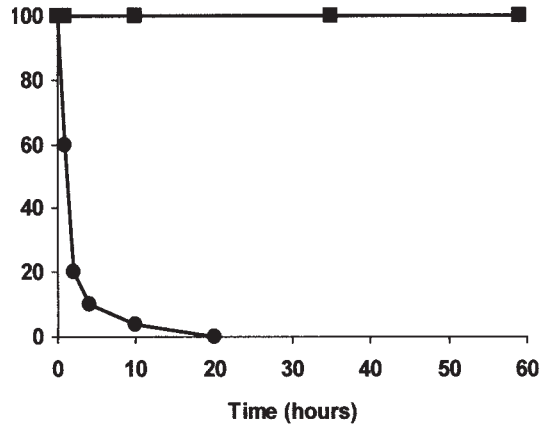

Fig. 6. Thermostability of different preparations of *C. antarctica B* lipase. Inactivation was performed at 50°C, pH 7.0. Octadecyl-Sepabeads (■), soluble enzyme (●).

activation of lipases. In this way, the rate and intensity of adsorption of different lipase isoforms on tailor-made hydrophobic supports may be dramatically different. In fact, the sequential adsorption of the crude of the *C. rugosa* lipase on agarose supports with different grade of hydrophobicity, butyl-agarose (low hydrophobicity), phenyl-agarose (medium hydrophobicity), and octyl-agarose (high hydrophobicity) permitted to separate the different isoforms of this lipase ***(20)***.

3.12. Reversibility of the Lipase Adsorption

Although it is interesting that the adsorption strength of the lipase to the supports was very high to prevent the desorption of the lipase under industrially rel-

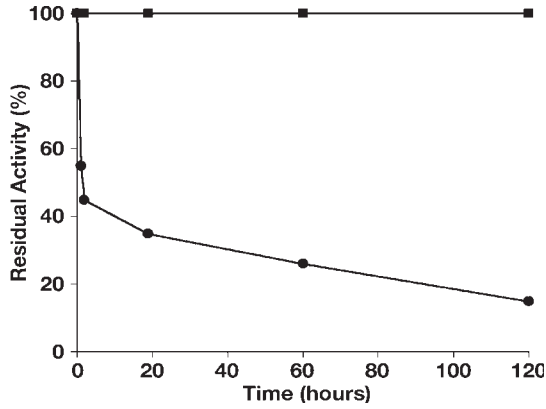

Fig. 7. Stability of different preparations of *R. miehei* lipase against cosolvents. Inactivation was performed at 25°C, pH 7.0, in 40% Dioxane. Octadecyl-Sepabeads (■), soluble enzyme (●).

Table 1
Desorption of the Immobilized Lipases From Octyl-Agarose or Octadecyl-Sepabeads Support

Lipase	Support	Triton X-100 (%)
C. antartica B	Octyl-agarose	1.00
	Octadecyl-Sepabeads	4.00
C. rugosa	Octyl-agarose	0.50
	Octadecyl-Sepabeads	3.00
Mucor miehei	Octyl-agarose	0.07
	Octadecyl-Sepabeads	0.20

Note: The numbers express the amount of detergent necessary to desorb 100% of lipase from the support.

evant conditions, it is convenient that the enzyme can, after enzyme deactivation, be desorbed from the support in order to reload the support with fresh enzyme and take advantage of the reversibility of the technique. **Table 1** shows that the adsorbed lipases on octadecyl-Sepabeads or octyl-agarose could be fully desorbed by using moderate concentrations of detergent. Moreover, the incubations with urea and guanidine enable the full desorption of the lipase.

The regenerated supports could be used to immobilize new batches of lipase without significant differences in terms of loading capacity compared to the first immobilization.

4. Notes

1. To take suspension the tips have to be cut.
2. In some instances, if immobilization is slow, it may be convenient to dilute the lipase preparation at low ionic strength (even adding some glycerol).
3. Check that desorption of the lipase caused by organic solvents is not produced.
4. It is first necessary to check the effect of a similar amount of organic solvent during the assay in the enzyme activity; organic solvents may be inhibitors or activator of the lipases.

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14

Immobilization and Stabilization of Proteins by Multipoint Covalent Attachment on Novel Amino-Epoxy-Sepabeads®

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Summary

The prospects of a new commercially available support (amino-epoxy-Sepabeads®) for enzyme immobilization are discussed in this chapter. These supports have a layer of epoxy groups over a layer of ethylenediamine that is covalently bound to the support. Thus, the support has a high anionic exchanger power and a high number of epoxy groups. Some relevant properties of this support are (1) immobilization proceeds at low ionic strength using amino-epoxy-Sepabeads, (2) immobilization is much more rapid using amino-epoxy supports than employing conventional epoxy supports, and (3) stability of the immobilized enzyme has been found to be much higher using the new support than in preparations using the conventional ones in many cases. Therefore, this support may be a good complement to the traditional hydrophobic epoxy supports.

Key Words: Heterofunctional supports; ionic adsorption plus multipoint covalent attachment; third generation of epoxy supports.

1. Introduction

Epoxy-activated supports are almost ideal matrices to perform very easy immobilization of proteins and enzymes at both laboratory and industrial scale ***(1–6)***. These activated supports are very stable during storage and during suspension in neutral aqueous media. Hence, they are easily handled before and during immobilization processes as well as over the long term. Furthermore, epoxy-activated supports are able to directly form very stable covalent linkages with different protein groups (e.g., amino, thiol, phenolic) under very mild experimental conditions

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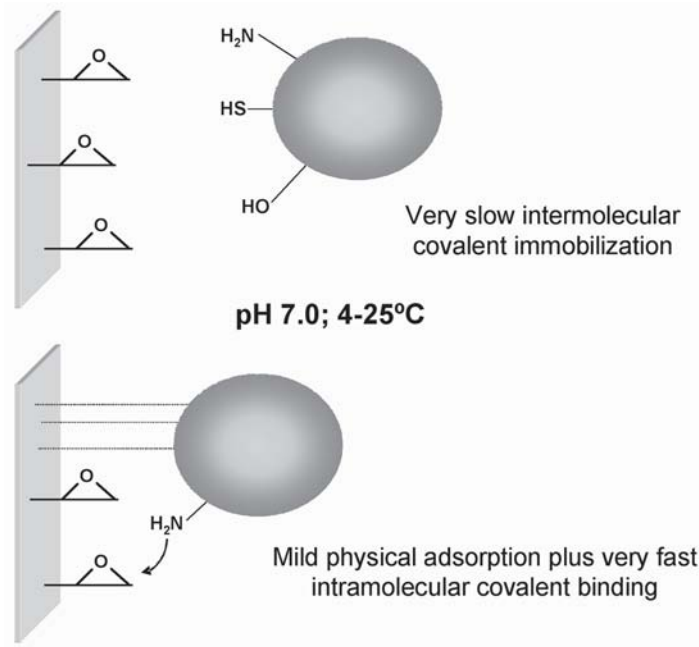

Fig. 1. Mechanism of immobilization of proteins on epoxy supports.

such as pH 7.0. The immobilization follows a two-step mechanism: first, a rapid mild physical adsorption between the protein and the support is produced and then a covalent reaction between the adsorbed protein and the epoxy groups occurs ***(5–11)*** (*see* **Fig. 1**).

However, the immobilization onto conventional epoxy supports has several problems:

1. Use of high ionic strength, which can affect the stability of certain enzymes.
2. The enzymes require a hydrophobic area to be immobilized on the support.
3. The enzymes will be immobilized by their hydrophobic area, which may be not the most convenient to stabilize the enzyme.
4. Hydrophobic supports are required for this immobilization, with a likely negative effect on the enzyme stability.

1.1. Multifunctional Epoxy Supports for Protein Immobilization

Bearing in mind this two-step mechanism for covalent immobilization of proteins on epoxy supports, multifunctional epoxy supports have been proposed as a second generation of activated supports that are able to covalently immobilized enzymes, antibodies, or other molecules under very mild experimental conditions. These multifunctional supports should contain two types of functional groups: (1) groups that are able to promote the physical adsorption of proteins (such as ionic

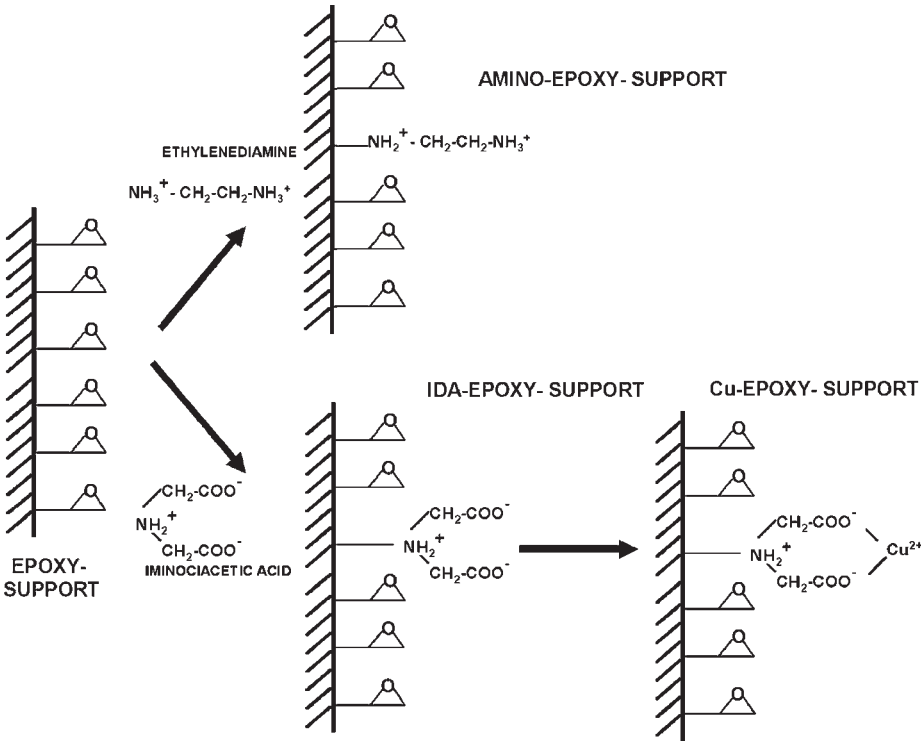

Fig. 2. Some different multifunctional epoxy supports for protein immobilization.

exchangers or by metal-chelate adsorption), and (2) groups that are able to covalently immobilize the enzymes such as epoxyde groups ***(12–17)***. The partial modification of the epoxy supports with ethylenediamine, iminodiacetic acid, metal chelates, or *m*-amino-phenyl-boronic acid (*see* **Fig. 2**) may be good alternatives for reaching this goal.

1.2. Third Generation of Epoxy Supports

Use of the first heterofunctional supports required substitution of some epoxy moieties by groups that could promote the physical adsorption of the proteins to the support. This resulting decrement in the number of epoxy groups available for the covalent multipoint immobilization compromised the desired solutions (*see* **Fig. 3**) ***(12)***. To avoid this, Residion srl (Milano, Italy) has developed a third generation of epoxy supports, in which the epoxy moieties have been introduced by attaching them to ethylenediamine (ED) covalently bound to the support surface (*see* **Fig. 4**) ***(16,17)***. Therefore, using these supports, there is a 1:1 ratio between the number of amino groups and the reactive epoxy groups, which determine the physical adsorption and covalent immobilization rate, respectively.

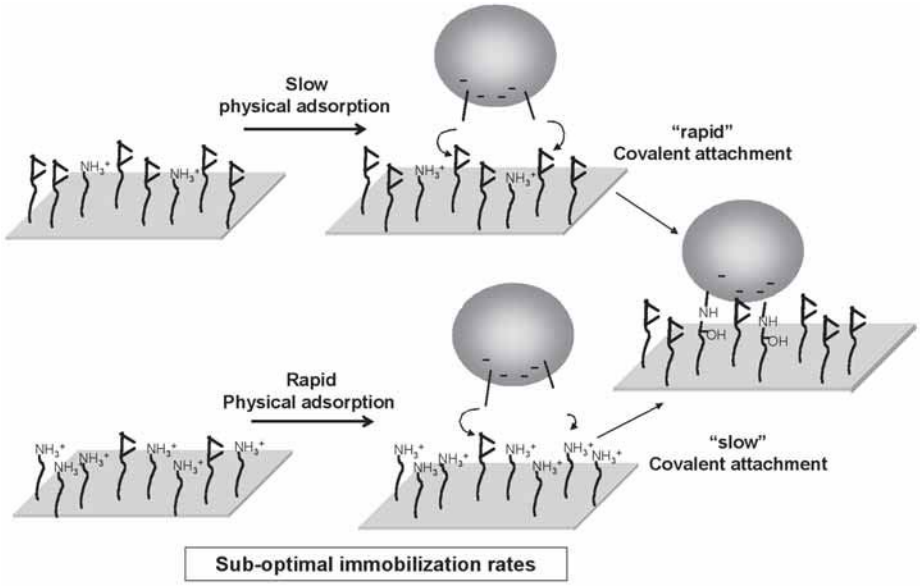

Fig. 3. Covalent immobilization of enzymes on epoxy-amine support obtained by modification of a small fraction of the epoxy groups contained in the support.

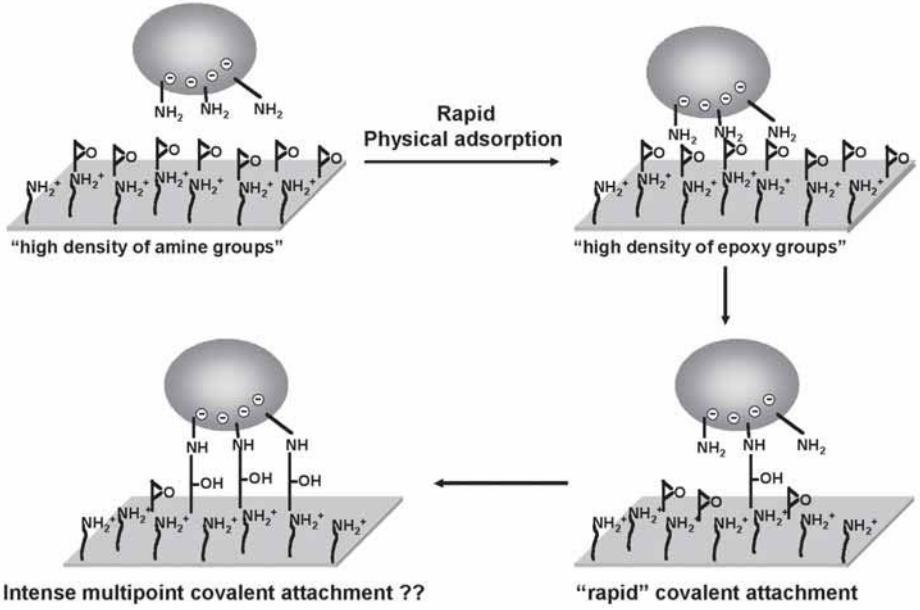

Fig. 4. Covalent immobilization of enzymes on epoxy-amine supports obtained by modification of a layer of ethylenediamine with epoxy moieties covalently bound to the support surface.

2. Materials

1. Commercial EC-HFA (new amino-epoxy supports) was purchased from Resindion SRL (Milan, Italy; *see* **Note 1**).
2. Immobilization buffer: 5 m*M* sodium phosphate buffer, pH 7.0.
3. Incubation buffer: 5 m*M* sodium bicarbonate buffer, pH 9.0 to 10.0.
4. Blocking solution: 3 *M* glycine, pH 8.5.
5. Desorption buffer: 1 *M* sodium phosphate, pH 7.0.

3. Methods

3.1. Preparation of Amino-Epoxy Supports and Enzyme Solution

1. Wash the support 10 times with five volumes of 5 m*M* sodium phosphate buffer, pH 7.0, at 4°C using a Büchner flask with glass-sintered funnel connected to a vacuum line.
2. Dissolve the proteins in 5 m*M* sodium phosphate, pH 7.0, take a sample as reference blank and test the enzyme activity (*see* **Note 2**).

3.2. Immobilization of Proteins on Amino-Epoxy Supports

1. Add the support to the enzyme solution prepared as described in **Subheading 2.**, **step 1**.
2. Gently stir the suspension (enzyme and gel) at 25°C (*see* **Note 3**).
3. Periodically, samples of supernatant and suspension were taken for assay of enzyme activity. Supernatant was achieved by using a tip filter or by centrifugation of the suspension (*see* **Note 4**).
4. Assay the enzyme activity of reference solution at the same time intervals as in **step 3**. The immobilization process is finished when the activity of the supernatant is zero.

3.3. Desorption of Proteins Noncovalently Immobilized on the Support

1. 2.5 mL of enzymatic suspension were taken and dried by filtration under vacuum.
2. The dried support was resuspended in 2.5 mL of 1 *M* sodium phosphate buffer, pH 7.0. The suspension was gently stirred for 30 min at 20°C.
3. The enzyme activity or the protein concentration of the supernatant was checked.
4. If there are no proteins released, covalent attachment was considered.

3.4. Multi-Interaction Step on Enzyme Immobilization

1. Then, the immobilized preparation was washed five times with three volumes of 5 m*M* sodium phosphate solution and then resuspended in three volumes of 100 m*M* sodium bicarbonate buffer, pH 9.0 to 10.0. Stirring is not necessary in this step.
2. The immobilized protein was left to interact with the support for different times (ranging from 1 d to 1 wk) before incubation with 3 *M* glycine, pH 8.5. Activity of the immobilized preparations could be followed all along the incubation (i.e., every day; *see* **Note 5**).

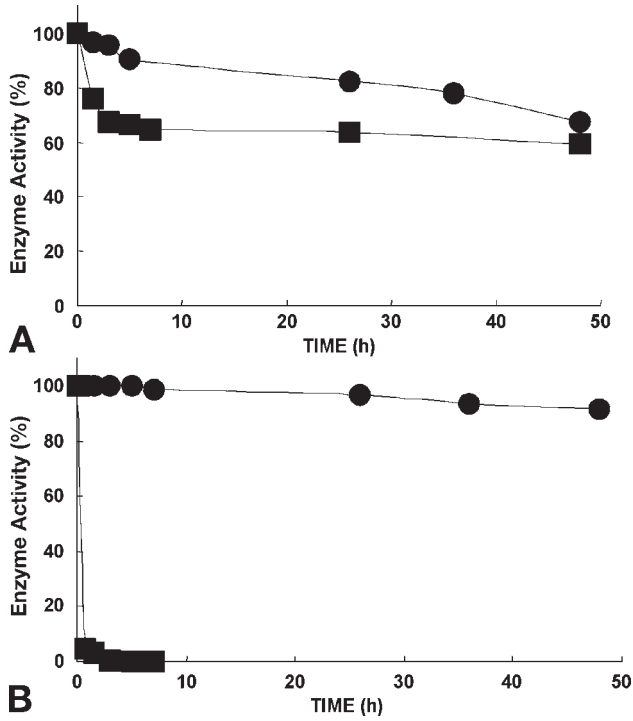

Fig. 5. β-galactosidase from *A. oryzae* immobilization on Sepabeads EC-EP1 (**A**) and Sepabeads EC-HFA (**B**) supports: (●) activity of the suspension; (■) activity of the supernatant. The enzyme was immobilized in presence of 1 *M* sodium phosphate when using Sepabeads EC-EP1 and 5 m*M* sodium phosphate for Sepabeads EC-HFA. All the enzyme immobilizations were carried out at 20°C, pH 7.0) as described in **Subheading 3**.

3.5. Blocking of Epoxy Groups of the Supports

1. The immobilized preparations were vacuum dried and resuspended in three volumes of 3 *M* glycine, pH 8.5, and stirred gently at 20°C for 8 h.
2. Finally, the enzyme preparation was washed with distilled water and stored at 4°C.

3.6. Immobilization of β*-Galactosidase From* A. oryzae *on Sepabeads® EC-HFA1*

1. Dissolve 500 mg of solid β-galactosidase (Sigma, grade XII) in 90 mL of 5 m*M* sodium phosphate solution, pH 7.0.
2. Assay the catalytic activity of this solution. Add 10 g of Sepabeads EC-HFA1 and assay the enzyme activity of both suspension and supernatant after 15 min. If any activity remains in the supernatant, stir the suspension for an additional 15 min, then repeat again the enzyme assays (*see* **Fig. 5**).
3. Stir this suspension very gently for 24 h at 25°C.
4. After this, evaluate covalent immobilization as described earlier (*see* **Subheading 3.3.**).

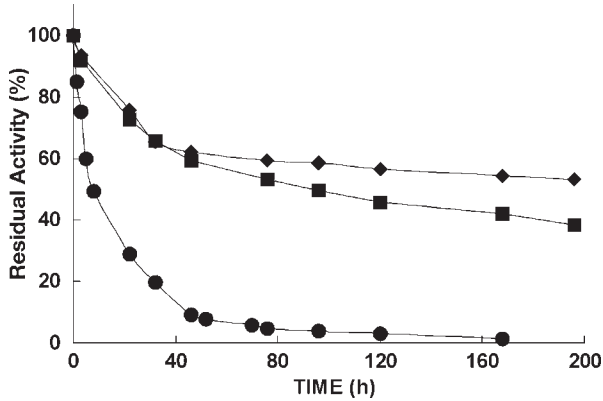

Fig. 6. Thermal stability of different β-galactosidase preparations covalently immobilized on Sepabeads EC-HFA. (●) Soluble enzyme. The enzyme was immobilized in 5 m*M* sodium phosphate, pH 7.0 at 20°C for 24 h and then blocked with glycine (■) or the pH was adjusted at 10.0 and the reaction enzyme-support reaction was left to proceed for 8 h before blocking (◆). Inactivation of enzyme derivatives was carried out at 55°C, pH 4.5.

5. Increase and adjust the enzyme suspension at pH 10.0 and gently stir the enzyme-support reaction for 8 h (*see* **Note 5** and **Fig. 6**).
6. Filter the suspension and then block the epoxy groups as described in **Subheading 3.5.**, **step 1**.
7. Then wash and filter the suspension with 25 m*M* phosphate buffer, pH 7.0, and the distilled water as described in **Subheading 3.5.**, **step 2**. Finally, filter it to dryness

3.7. Immobilization of Glutaryl Acylase on Sepabeads EC-HFA2

1. Mix 5 mL of a glutaryl acylase (GA) from Roche with 20 mL of 5 m*M* sodium phosphate solution, pH 7.0.
2. Assay the catalytic activity of this solution. Add 10 g of Sepabeads EC-HFA2 to 20 mL of the previous GA and assay the enzyme activity of both suspension and supernatant after 15 min. If any activity remains in the supernatant, stir the suspension for an additional 15 min, then repeat again the enzyme assays (*see* **Fig. 7**).
3. Stir this suspension very gently for 24 h at 25°C.
4. After this, evaluate covalent immobilization as described above (*see* **Subheading 3.3.**)
5. Increase and adjust the enzyme suspension at pH 10.0, gently stir the enzyme-support reaction for 7 d at 4°C (*see* **Note 5**). (*See* effect on enzyme stability in **Fig. 8**.)
6. Filter the suspension and then block the epoxy groups as described in **Subheading 3.5.**, **step 1**.
7. Wash and filter the suspension with 25 m*M* phosphate buffer, pH 7.0, and the distilled water as described in **Subheading 3.5.**, **step 2**. Finally, filter it to eliminate all inter-particle water.

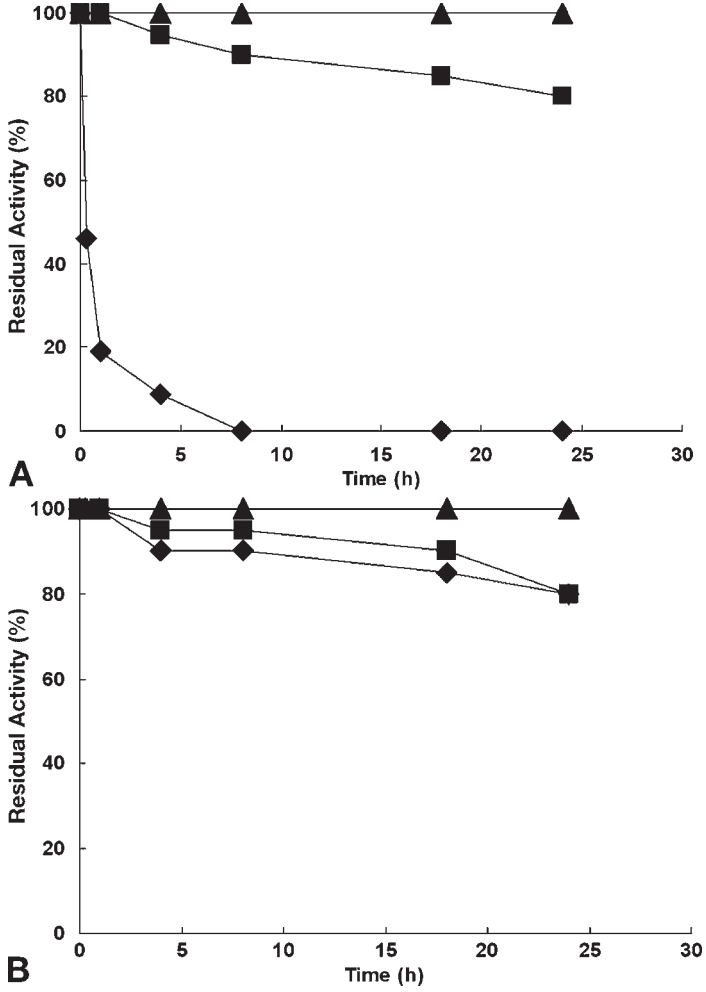

Fig. 7. Immobilization course of GA onto EC-HFA2 at different ionic strenghs. (**A**) Immobilization was carried out in 25 m*M* potassium phosphate buffer at 25°C, pH 7.0. (◆) Supernatant. (■) Suspension. (▲) Control (soluble enzyme in immobilization conditions). **(B)** Immobilization was carried out in 1*M* potassium phosphate buffer at pH 7.0. (◆) Supernatant. (■) Suspension. (▲) Control (soluble enzyme in immobilization conditions).

4. Notes

1. Sepabeads EC-EP is a commercially available epoxy support with different pore sizes (Resindion SRL) and epoxy groups concentration (EC-EP1 [little], EC-EP2 [medium], and EC-EP3 [large]).
2. If it is necessary, verify reference enzyme activity to evaluate yield and recovery activity of enzyme immobilization process.

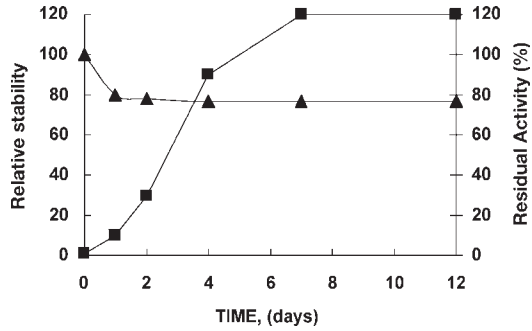

Fig. 8. Effect of the incubation at alkaline pH value (before blocking the remaining epoxy in the support) on the stability of immobilized glutaryl acylase EC-HFA2**.** The derivative was incubated in 25 m*M* sodium bicarbonate at 4°C, pH 10.0 (▲) Suspension. All derivatives were thermally inactivated at 45°C in potassium phosphate buffer, pH 7.0, and the relative stability was defined as the ratio between the half-lives of the derivatives and soluble enzyme, respectively (▲). The percentage of lost activity during the incubation at pH 10.0 (■) was analyzed at different times.

3. Amounts, kind of EC-HFA support ratios Vgel/Vsuspension used, and reaction times must be established for each enzyme. Avoid magnetic stirring to reduce abrasion of the support.
4. If the enzyme activity is decreased because of enzyme inactivation it must be distinguished from loss in enzyme activity of the supernant resulting from immobilization process.
5. Multipunctual derivatives can be reached through long-time incubation of enzyme suspension after the immobilization has concluded. Additional bonds may be formed by keeping the suspension at pH 10.0. The optimum multi-interaction time required for each enzyme must be established in every case. For this purpose, it is necessary to prepare enzyme derivatives with different multi-interaction times and check the thermal stability of each. The time of choice is a compromise between the shortest time that provides both an optimal stability and enzyme recovery.

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Improved Stabilization of Chemically Aminated Enzymes Via Multipoint Covalent Attachment on Glyoxyl Supports

Tamara Montes, Fernando López-Gallego, Manuel Fuentes, Cesar Mateo, Valeria Grazu, Lorena Betancor, Jose M. Guisan, and Roberto Fernandez-Lafuente

Summary

Chemical modification and immobilization of proteins have been usually utilized as parallel techniques to improve enzyme stability. In this chapter, we show that chemical modification of the protein surface to greatly increase its reactivity with the groups of a support activated with glyoxyl residues may be a very good alternative for greatly increasing the protein stability via multipoint covalent attachment. For this purpose, some of the carboxylic acids of the proteins are transformed into amino groups by reaction with ethylendiamine via the carbodiimide coupling method. The new amino groups have a lower pK than Lys residues, enabling immobilization under milder conditions and a higher degree of stabilization. These results show that the coupling of different stabilization techniques may yield a synergistic effect, higher than any individual strategy.

Key Words: Enzyme stability; protein surface modification; multipoint covalent attachment; glyoxyl supports.

1. Introduction

Enzyme stabilization is still one of the critical issues in the design of a biotransformation ***(1,2)***. Enzyme inactivation usually starts with reversible conformational changes that finally promote their irreversible inactivation ***(3)***. Thus, most of the strategies for protein stabilization are focused on the prevention of the first three-dimensional (3D) distortion of the protein. Multipoint covalent attachment may be one of the techniques that have offered the best results for solving this problem. It has been suggested that this behavior could result from a more rigid conformation generated by numerous bonds linking the enzyme to the insoluble carrier, which could prevent the conformational changes that may vary the relative

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position of the residues implicated in the immobilization. Glyoxyl supports have been described as a very adequate immobilization system to yield immobilized-stabilized proteins via multipoint covalent attachment. Many enzymes have been stabilized using this technique—e.g., penicillin G acylase from *Escherichia coli* ***(4)*** and *Kluyvera citrophila* ***(5)***, trypsin ***(6)*** chymotrypsin ***(7)***, alcalase ***(8)***, carboxypeptidase A ***(9,10)***, FNR NADP-reductase ***(11)***, esterase ***(12)***, thermolysin ***(13)***, DAAO ***(14)***, catalases ***(15,16)***, and lipases from different sources ***(17,18)***, urokinase ***(19)***, L-aminoacylase ***(20)***, Chitosinase ***(21–23)***.

These good results are the consequence of some glyoxyl-agarose properties, namely, low-steric hindrances for the amino–aldehyde reaction and a short spacer arm that renders high stability at longer reaction times ***(24)***. These properties are shared with other groups, like the epoxy ones ***(25)***, but glyoxyl supports give in general higher stabilization factors, suggesting some other peculiarity that is important to obtaining this very high degree of stabilization.

Glyoxyl agarose has an apparent drawback as the need of alkaline pH values to achieve a multipoint covalent attachment in the immobilization process and the necessity of using highly activated supports, because of the reversibility of the individual shift's base links that makes that the enzyme only become immobilized via a certain multipoint covalent attachment ***(26)***.

However, this apparent problem is what causes glyoxyl-supports to reach such high-stabilization values when applied to most enzymes. It promotes the "auto-oriented" immobilization of the protein by the area/s with the highest density/ies of lysines, where the most intense multipoint attachment may take place ***(26)***.

Moreover, the immobilization conditions need to be carefully designed to permit a very intense multi-interaction. They must exhibit good support–enzyme geometrical congruence, moderately high temperature, or high density of reactive groups ***(5)***.

It has been suggested recently that the stabilization of enzymes achieved via multipoint covalent attachment on glyoxyl support may be improved if other tools, such as chemical or genetic modification, are used to fulfill this goal ***(27,28)***. The genetic additional enrichment in Lys of some very rich areas in Lys of penicillin G acylase permits to greatly improve the immobilized PGA stability ***(27)***.

An alternative approach could be to chemically modify the enzyme, not to get a more stable enzyme, but to have a protein surface very enriched in reactive groups and, therefore, with much better possibilities of yielding a more intense multipoint covalent attachment during immobilization **(***see* **Fig. 1) *(28)***.

In this chapter, we will propose the use of the chemical amination of the enzyme surface to improve the possibilities of achieving a more intense multipoint covalent attachment

2. Materials

1. Cross-linked 10BCL and 6BCL agarose beads were donated by Hispanagar S.A. (Burgos, Spain).
2. Protein amination solution was 1 *M* ethylenediamine (ED), pH 4.7, containing different amounts of 1-ethyl-3-(dimethylamino-propyl) carbodiimide (EDAC) (*see* **Note 1).**

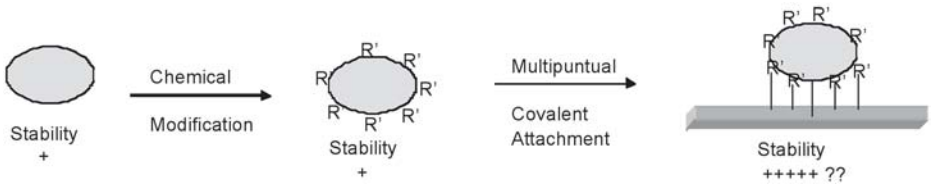

Fig. 1. Chemical modification for achieving a more stable immobilized preparation.

3. Glycidol (2,3-epoxy propanol) was purchased from Sigma-Aldrich (St. Louis, MO; s*ee* **Note 2**).
4. Support oxidation solution: 0.1 *M* sodium periodate in water.
5. Protein immobilization buffer: 0.1 *M* bicarbonate buffer, pH 10.0; s*ee* **Note 3)**.

3. Methods

3.1. Enzyme Amination

1. Add 5 mL of 11 mg/mL soluble enzyme to 45 mL of protein amination solution **(s***ee* **Note 4**).
2. Stir gently for 90 min at 25°C and then add 10 mL of a 0.5 *M* hydroxylamine solution, pH 7.0.
3. Dialyze the enzyme solution five times with 50 volumes of a 25 m*M* potassium phosphate buffer and store at 4°C.

3.2. Preparation of Glyoxyl-Supports

3.2.1. Activation of Agarose Gel to Glyceryl-Agarose

1. Wash 105 g (150 mL) of commercial agarose 6BCL or 10BCL thoroughly with distilled water (s*ee* **Note 5**).
2. Suspend the agarose in distilled water up to a total volume of 180 mL.
3. Add 50 mL of 1.7 *N* NaOH solution containing 3.4 g of sodium borohydride to this suspension.
4. Take the vessel to an ice bath; while gently stirring add dropwise 36 mL of glycidol (s*ee* **Note 6**).
5. Gently stir the suspension for 18 h at 25°C.
6. Filter and wash the support thoroughly with distilled water.

3.2.2. Oxidation of Glyceryl-Agarose to Glyoxyl-Agarose

1. Resuspend 105 g of glyceryl-agarose (*see* **Subheading 3.2.1.**) in 1500 mL of distilled water.
2. Add the proper amount of support oxidation solution slowly to this suspension while stirring (s*ee* **Note 7**).
3. Gently stir the suspension for 2 h.
4. Wash the support with an excess of distilled water and filter it to dryness.

3.3. Enzyme Immobilization

1. Incubate 10 g glyoxyl-agarose with 100 mL of chemically aminated or nonmodified enzyme solution prepared in immobilization buffer.
2. Add 1 mL of distilled water to 9 mL of enzyme solution to be used as reference solution (*see* **Note 8**).
3. Gently stir the mixtures by end-over-end rotation at 25°C.
4. Aliquots of supernatant and suspension were withdrawn at regular time intervals (*see* **Note 9**) to assay enzyme activity.
5. Assay the activity of the reference solution using the same time intervals and aliquot volumes as in **step 4**.
6. The immobilization process finishes when activity of the supernatant is zero.
7. Then, the immobilized preparation was washed five times with three volumes of immobilization buffer.

3.4. Structural Stabilization by Multipoint Covalent Attachment

1. Resuspend the enzyme derivative (*see* **Subheading 3.3.**) in 100 mL of immobilization buffer.
2. Maintain the suspension for several hours (*see* **Note 10**). Although stirring is not necessary because the enzyme is already immobilized on the support, stirring is necessary to measure the activity of the suspension.
3. Add 100 mg of solid sodium borohydride and stir the suspension for 30 min at 25°C.
4. Finally, wash the enzyme derivative with 25 m*M* sodium phosphate, pH 7.0, while vacuum filtering to eliminate the borohydride.
5. Enzyme derivatives are stored at 4°C.

3.5. Improving the Stabilization of PGA by Multipoint Covalent Attachment on Glyoxyl Supports Via Partial Amination of the Protein Surface

3.5.1. Enzyme Amination

1. 5 mL Soluble Penicillin G Acylase (PGA) from *Escherichia coli* was incubated with 45 mL of protein amination solution containing EDAC to a final concentration of 10^{-3} *M*. This protocol of amination allows the modification of between 40 and 50% of the external carboxylic groups. This means that the modified enzyme has added to the 41 amines from the Lys, 12–14 new amino groups were generated via the amination. The modification presented negligible effect in the enzyme activity, but decreased the stability of the enzyme (by a factor of eightfold). However, as the full modification of PGA carboxylic groups caused a greater destabilization, the partial modification of the carboxylic groups with ED was chosen to carry out further studies.

3.5.2. Penicillin G Acylase Immobilization

1. 10 g Glyoxyl agarose 10 BCL were added to 100 mL of aminated or nonmodified PGA solution (0.55 mg protein/mL) in 100 m*M* sodium bicarbonate buffer, pH 9.0 to 10.0, containing 100 m*M* phenylacetic acid and 20% glycerol ***(4)***. The

Table 1
Immobilization of Soluble Modified PGA Onto Glyoxyl-Agarose at pH 9.0[a]

Enzyme	Modification	Yield of immobilization after 4 h (%)
PGA	Nonmodified	0
	Aminated	100

[a]PGA modified with 1 *M* ED and 10^{-3} m*M* EDAC at pH 4.75.

suspension was then gently stirred for 3 h at 25°C, pH 10.0 (*see* **Note 11**).

2. When immobilization was performed at pH 10.0, full immobilization of PGA was observed within the first moments, using both nonmodified and aminated PGA. Residual activity was near 100% in both cases.

   At pH 9.0, it was not possible to immobilize the nonmodified enzyme. Only the aminated enzyme could be rapidly and fully immobilized on the support (**Table 1**). This fact could be explained because the pK value of ε-NH_2 of external lysines is 10.7, whereas the pK value of the artificially introduced primary amino groups is 9.2; therefore, they are even more reactive at pH 9.0 than the superficial lysines at pH 10.0.
3. The activity of both immobilized and soluble PGA was measured as follows: the initial reaction rates were measured using an automatic titrator (DL50 Mettler Toledo) to determine the amount of phenylacetic acid formed. The assays were carried out by adding aliquots of PGA to 10 mL of a 10 m*M* penicillin G in 0.1 *M* sodium phosphate/0.5 *M* NaCl, pH 8.0, at 25°C. The reaction mixture was titrated with 100 μ*M* NaOH, stored at 25°C, and mechanically stirred.

   One international unit (U) of PGA activity was defined as the amount of enzyme that hydrolyzes 1 mmol of penicillin G/min at pH 8.0 at 25°C.

3.5.3. Structural Stabilization Via Multipoint Covalent Attachment

1. The aminated PGA immobilized on glyoxyl agarose at pH 10.0, presented a half life around two times higher than the unmodified immobilized PGA (that is 10,000-fold more stable than the one point immobilized PGA) ***(4)*** (*see* **Fig. 2**).
2. The stability of the derivatives immobilized at pH 9.0 was lower than the ones immobilized at pH 10.0. However, the stability was greatly improved if, after immobilization at pH 9.0, the pH value was increased at pH 10.0 to favor the reaction of the lysines of the protein surface with the aldehyde groups on the support. This derivative was found to be around twice as stable than the aminated PGA directly immobilized at pH 10.0 (*see* **Fig. 3**). At pH 10.0, the enzyme is immobilized by the region/s with the highest density of lysines plus primary amino groups introduced by the chemical modification, whereas at pH 9.0, the immobilization proceeds through the area with the highest density of primary amino groups introduced by chemical modification. This different orientation may permit a higher number of enzyme support links to be established and/or the implication in the immobilization of a region critical in the inactivation of the enzyme.

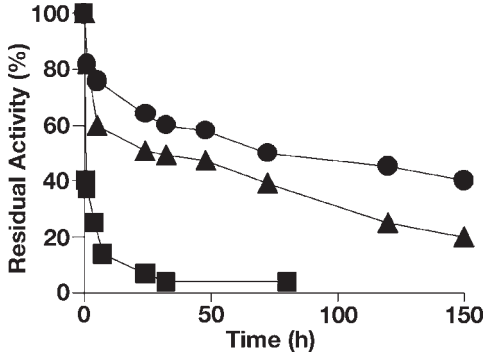

Fig. 2. Thermal inactivation of different PGA glyoxyl derivatives immobilized at pH 10.0. Thermal inactivation was carried out at 66°C, pH 7.0. (▲) PGA immobilized onto glyoxyl-agarose; (■) PGA immobilized on glyoxyl-agarose and then modified with 1 *M* ED and 10^{-3}*M* EDAC; (●) PGA modified with 1 *M* EDA in the presence of 10^{-3} *M* EDAC and after immobilized onto glyoxyl-agarose.

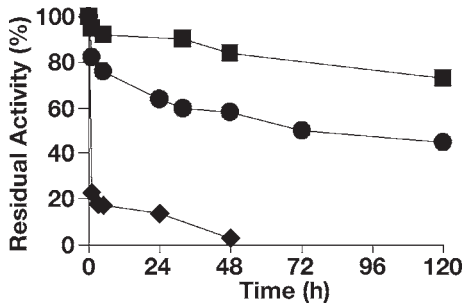

Fig. 3. Thermal inactivation courses of PGA derivatives immobilized at different pH values. (◆) Aminated PGA immobilized at pH 9.0 onto glyoxyl-agarose; (●) aminated PGA immobilized at pH 10.0 onto glyoxyl-agarose; (■) aminated PGA immobilized at pH 9.0 onto glyoxyl-agarose and incubated at pH 10.0 for 3 h.

3.6. Improving the Stabilization of Glutaryl Acylase by Multipoint Covalent Attachment on Glyoxyl Supports Via Partial Amination of the Protein Surface

3.6.1. Enzyme Amination

1. 5 mL Soluble glutaryl 7-ACA acylase (GA) from *Pseudomonas* spp. was added to 45 mL of 1 *M* ED, pH 6.0, containing 10 m*M* of EDAC (*see* **Note 12**). Because of enzyme inactivation the amination process was carried out at pH 6.0 instead of pH 4.7. This yields a modification of the carboxylic groups between 40 and 50%, which means that about 15 amino groups were added to the only 9 Lys presented in the surface of GA. The activity of the aminated enzyme decreased 20%. However, the modification presented a negligible effect on the enzyme stability.

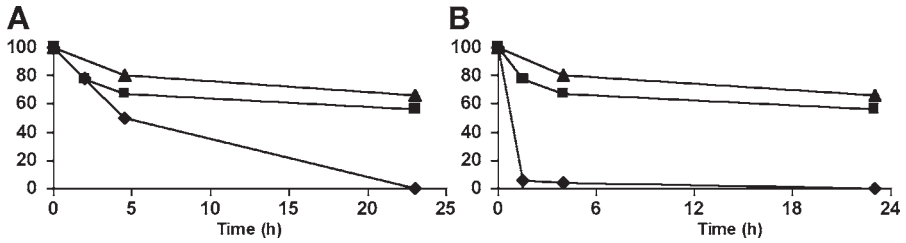

Fig. 4. Immobilization course of nonmodified GA (**A**) and aminated GA (**B**) onto glyoxyl-agarose at pH 10.0. ◆, Supernatant; ■, suspension; ▲, reference suspension (*see* **Subheading 3.3.**). Immobilization was performed in 100 m*M* sodium carbonate buffer, pH 10.0 at 25°C.

3.6.2. Enzyme Immobilization

1. 10 g Glyoxyl agarose 6 BCL was added to 100 mL of a GA solution (10 mL of aminated or nonmodified GA in 90 mL of 0.1 *M* potassium bicarbonate buffer [pH 9.0 or 10.0]). The suspension was then gently stirred for 24 h at 25°C, pH 10.0 (*see* **Note 11**).

   When the immobilization was carried out at pH 10.0, the unmodified enzyme showed a very slow immobilization rate onto glyoxyl agarose support (50% of the immobilization was achieved after 4 h), whereas the modified GA was almost fully immobilized after 1 h (*see* **Fig. 4**). The slow immobilization rate of the nonmodified enzyme could be explained by the low amount of lysine residues in the surface of GA, critical when using a support like glyoxyl that requires a multipoint immobilization of the enzyme ***(24,26)***. The use of aminated GA, with around threefold more amino groups, permitted a much more rapid immobilization. The immobilization promoted a decrease in GA activity: by 50%, when using the nonmodified enzyme and by 40%, when using the aminated GA.

   At pH 9.0, it was not possible to immobilize the nonmodified enzyme. However, the aminated enzyme could be rapidly and fully immobilized on the support **(Table 2)**. This could be explained because the artificially introduced primary amino groups are more reactive at pH 9.0 than the superficial lysines.
2. Enzyme activity was evaluated using an automatic titrator (DL50 Mettler Toledo) to titrate the release of glutaric acid produced by the hydrolysis of 10 m*M* solution of glutaryl 7-ACA in 0.1 *M* potassium phosphate buffer, pH 7.0. A 25-m*M* NaOH solution was employed to titrate the reaction mixture.

   One unit of GA activity was defined as the amount of enzyme that is necessary to produce 1 µmol of glutaric acid/min in the previously described conditions.

3.6.3.Structural Stabilization Via Multipoint Covalent Attachment

1. The immobilization of the nonmodified GA enzyme on glyoxyl agarose at pH 10.0 improved its stability by 26-fold. Nevertheless, when the aminated enzyme was immobilized onto glyoxyl agarose at the same pH, the stabilization factor was fivefold higher than when prepared with the nonmodified enzyme (*see* **Fig. 5**).

Table 2
Immobilization of Soluble Modified GA Onto Glyoxyl-Agarose at pH 9.0 [a]

Enzyme	Modification	Yield of immobilization after 4 h (%)
GA	Nonmodified	0
	Aminated	94

[a]GA modified with 1 *M* ED in the presence of 10^{-2} *M* EDAC at pH 6.0.

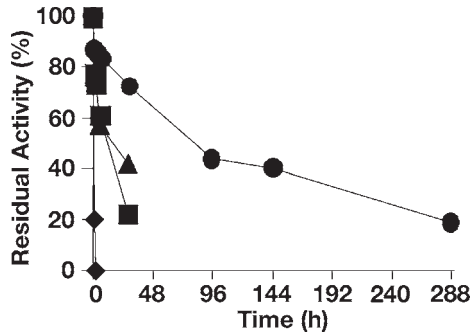

Fig. 5. Thermal inactivation of different GA glyoxyl derivatives prepared at pH 10.0. (▲) Glutaryl acylase immobilized on glyoxyl agarose; (■) glutaryl acylase immobilized on glyoxyl acylase and then modified with 1 *M* ED and 10^{-2} *M* EDAC; (●) glutaryl acylase modified with 1 *M* ED and 10^{-2} *M* EDAD and then immobilized onto glyoxyl agarose; (◆) soluble glutaryl acylase. Experiments were carried out at 45°C, pH 7.0).

2. The stability of the derivatives immobilized at pH 9.0 was lower than the ones in which the immobilization was carried out at pH 10.0. However, the stability was improved if, after immobilization at pH 9.0, the pH value was increased at pH 10.0, yielding a similar value to the enzyme directly immobilized at pH 10.0 (*see* **Fig. 6**).

4. Notes

1. It has been reported that the use of 0.1 *M* ED at pH 4.7 and 10 m*M* EDAC allows the full modification of the carboxylic groups of the protein surface, while using 1 m*M* EDAC in 1 *M* ED at pH 4.7 the modification degree is only between 40 and 50% ***(28)***.
2. Store between 0 and 5°C. **Caution:** toxic!
3. During immobilization process, some enzymes may be inactivated at alkaline pH. In these cases, some inhibitors may be added to the immobilization buffer to protect the enzyme.
4. The choice of the amount of EDAC to be used is done by evaluating its impact on the activity and stability of the previously immobilized enzyme. Thus, modification conditions which decrease the stability of the modified enzymes are avoided ***(28)***.

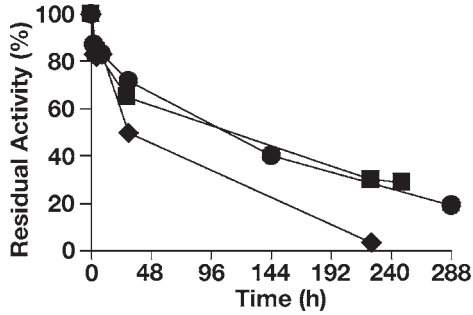

Fig. 6. Thermal inactivation courses of GA derivatives immobilized at different pH values. (◆) Aminated GA immobilized at pH 9.0 onto glyoxyl-agarose; (●) aminated GA immobilized at pH 10.0 onto glyoxyl-agarose; (■) aminated GA immobilized at pH 9.0 onto glyoxyl-agarose and incubated at pH 10.0 for 3 h.

5. Avoid magnetic stirring of agarose, especially during long reaction times.
6. Glycidol addition must be very slow to prevent the temperature rising over 25°C.
7. Oxidation of glycols with sodium periodate is a stoichiometric reaction. Therefore, the activation degree of the support can be easily controlled through the periodate concentration used. The protocol described enables an activation degree of 75 and 200 μmol/mL. To achieve this level, 112.5 and 300 mL, respectively, of oxidation solution have to be added to completely oxidize agarose 6 and 10BCL.
8. If the enzyme activity decreases during the course of immobilization resulting from enzyme inactivation, this effect must be distinguished from loss of the supernatant resulting from immobilization.
9. Supernatant was achieved by using pipet filter or by centrifugation of the suspension.
10. Rigidification of the protein structure via the formation of multipoint covalent linkages between its nonionic amine groups and the reactive groups of the support could be obtained by keeping the suspension at pH 10.0 for a fairly long interaction time at 25°C. The optimum multi-interaction time is the shortest one that provides the maximal stability of the enzyme derivative.
11. The already immobilized enzymes were incubated under the conditions reported to yield the maximum stability for the nonmodified enzyme ***(4)***.
12. Commercial preparation of GA (purchased from Roche) was diluted fivefold (v/v) in 25 m*M* potassium phosphate buffer, pH 7.0, and then dialyzed threefold against 100 volumes of 25 m*M* potassium phosphate buffer, pH 7.0. The dialyzed enzyme was then centrifuged (24,149*g* for 30 min at 4°C) and the supernatant (containing 16 U/mL and 11 mg of protein/mL) were used as the enzymatic preparation for further experiments. More than 90% of initial activity was recovered after this process.

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16

Stabilization of New Imprint Property of Glucose Oxidase in Pure Aqueous Medium by Cross-Linked-Imprinting Approach

Alankar Vaidya and Lutz Fischer

Summary

Bioimprinting is a well-known strategy to manipulate the catalytic properties of the enzymes. However, the lack of expression of the newly acquired imprinted property by an enzyme in aqueous surrounding is the chief limitation that restricts the application of this technique. In this chapter we used a combinatorial approach which amalgamates the benefits offered by traditional cross-link-immobilization technique (rigidity and stability) and bioimprinting approach (induced tailor-made catalytic property). This contemporary method is termed cross-linked-imprinting (CLIP). In order to extend the range of its application, glucose oxidase (GO; β-D-glucose:oxygen L-oxidoreductase, E.C. 1.1.3.4) was selected as a biochemically demanding enzyme (FAD-depending, tetrameric glycoprotein). Galactose, a competitive inhibitor of GO was taken as a template molecule during imprinting. It was demonstrated that the induced imprinted memory created for specific galactose–GO interactions was preserved during precipitation and the resultant modified active site was "frozen" by covalent cross-linking carried out in organic solvent. In separate following experiments, it was established that CLIP-GO not only binds galactose as a conventional ligand, but catalyzes the oxidation of galactose to galactono-1,4-lactone in aqueous medium which was not the case without CLIP method.

Key Words: Tailor-made enzyme catalysis; glucose oxidase; protein imprinting; bioimprinting; biochemical protein engineering; CLIP technique.

1. Introduction

It is known that catalytic properties of enzymes such as stability and substrate/enantio-selectivity could be modified by genetic engineering ***(1)***. This approach requires intricate multisteps such as identification and isolation of a particular gene, rational or random mutagenesis of the gene, expression of the recombinant gene in

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appropriate vector, and overexpression and screening of the recombinant enzyme using high-throughput assay. Not only are these steps laborious and time consuming, they also necessitate a lot of instruments and monetary investment. Hence, since 1990, intensive research efforts have been directed toward providing new alternatives for genetic engineering in order to develop better enzyme catalysts. Bioimprinting of enzymes is one of those alternatives that is simple to perform, less time consuming, and one of the cheapest chemical modification methods offering improved enzyme properties ***(2–4)***. The basic bioimprinting stratgy is outlined in **Fig. 1**.

Crude enzymes or proteins can be modified by bioimprinting to obtain tailor made enzymes or artificial antibodies. Bioimprinted enzymes that previously catalyzed a particular reaction is modified so as to demonstrate higher reaction rates in nonaqueous organic medium or to begin accepting the "stereo-counterpart" of the same substrate. This is illustrated in following examples: bioimprinting of α-chymotrypsin enhances selectivity during synthesis of an unusal substrate (i.e., D-form of ethyl ester of *N*-acetylated amino acids) ***(2)***, subtilisin lyophilized from aqueous solution in presence of its inhibitor *N*-acetyl-L-tyrosine amide demonstrates a 55-fold increase in transesterification reaction between *N*-acetyl-L-alanine methyl ester and propanol in anhydrous octane ***(5)***, and interfacial activation-based bioimprinting of lipases results in more than a two-magnitude increase in esterification and alcoholysis carried out in nonaqueous medium ***(3)***. Furthermore, the advantage of bioimprinting in modification of noncatalytic globular proteins (such as albumins) is to develop artificial antibodies, as exemplified in following: selective adsorbents of L-malic acid from bioimprinted bovine serum albumin recognizes a substrate with 10 times more selectivity in anhydrous organic solvents ***(6)***; and enzyme-like properties were induced in β-lactglobulin using *N*-isopropyl-4-nitrobenzyl-amine as an imprint molecule resulting in three times the enhanced β-elimination of 4-fluoro-4-[*p*-nitrophenyl] butane-2-one as compared to nonimprinted protein in dry acetonitrile ***(7)***.

Close examination of these examples, however, shows that the induced catalytic reactions performed by bioimprinted enzymes or proteins were carried out exclusively in nonaqueous dry organic solvents. This is the major flaw in the classical bioimprinting approach, within which the new memory (created in biomolecules during imprinting without stabilizing the modified conformation of protein) is erasable and sensitive to the water content of the medium ***(2,4,8)***. Furthermore, when a percentage of the water in the reaction medium increases it is concomitantly reflected in a decreased reaction rate. Increasing the water content of the medium results in greater flexibility and decreased rigidity of the modified active site of an enzyme, which apparently "erases" the imprint-induced conformational chages. Therefore, this chapter describes how to stabilize the bioimprinted memory in aqueous medium, the most common prevalent milieu for many enzymatic biochemical reactions.

As shown in **Fig. 1**, the formation of a complex between print molecule and enzyme takes place in aqueous solution. This complex is precipitated so that the transition state of the enzyme or protein bound to the print molecule is frozen in organic solvent. Thus, a modified imprinted active site is generated. Now, if this

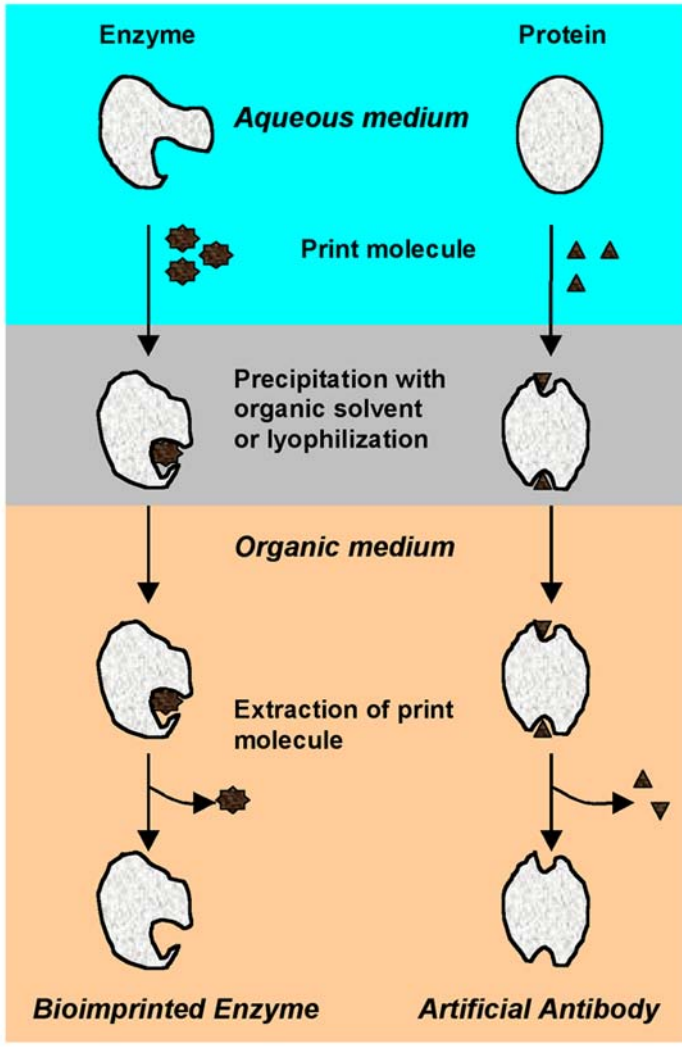

Fig. 1. The basic bioimprinting strategy.

modified biomolecule is placed in aqueous medium again then the imprinted active site will revert back to its native conformation and cease to demonstrate the acquired imprinted property. To restrict this flipping back of a modified active site to its native and thermodynamically favorable conformation we developed the combinatorial cross-linked imprinted protein approach (we termed it CLIP). The methodology is depicted in **Fig. 2**. Here, glucose oxidase (GO) is selected as an intricate model enzyme to modify its substrate selectivity (to see whether GO accepts galactose as its substrate in addition to its normal substrate, glucose) and thereby

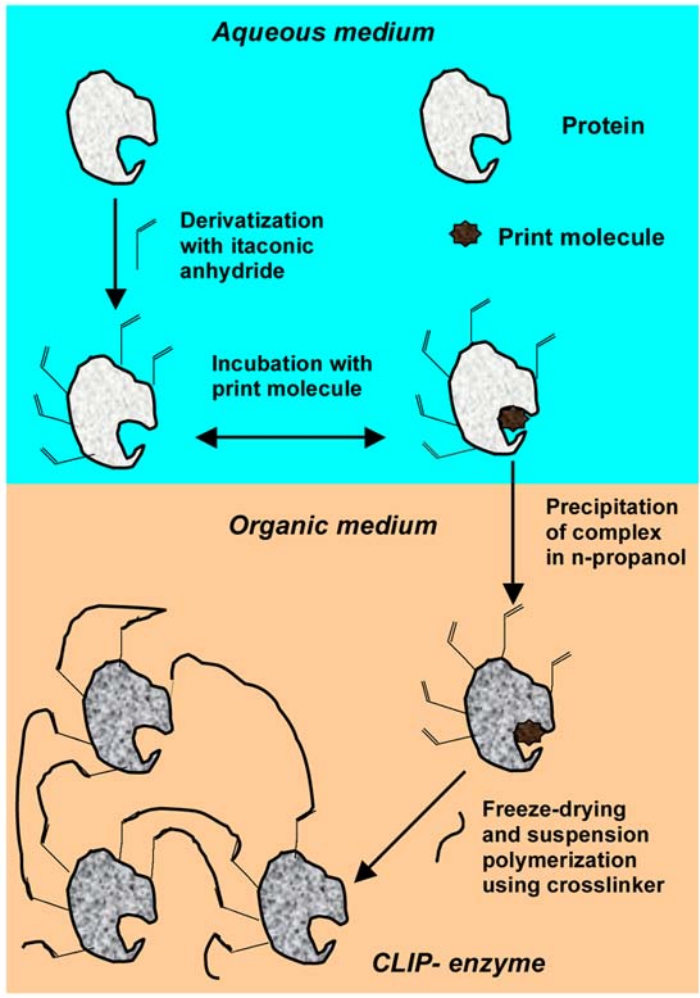

Fig. 2. CLIP methodology

to demonstrate the feasibility of the CLIP approach in inducing new catalytic properties. Further, the possibility of scaling-up the catalytic oxidation of galactose using CLIP-GO is described.

We previously demonstrated the enhanced stability of β-glucosidase using a two-step immobilization technique *(9)*. In the present CLIP method a similar immobilization procedure is adopted, wherein the first step is derivatization using itaconic anhydride to introduce polymerizable vinyl groups into GO. It is important to optimize the percentage of derivatization in order to maintain or increase the specific activity of native enzyme. Then, after purification of the derivatized GO using size-exclusion chromatography, the resulting dried enzyme is used for imprinting. D-Galactose, a competitive inhibitor of GO, is used as a print molecule

and imprinting is carried out in aqueous medium. The GO–galactose complex is precipitated using 1-propanol. Subsequently, the freeze-dried precipitate is suspended in dry anhydrous cyclohexane as a porogenic solvent and chemically cross-linked using excess of cross-linking agent. This excess amount is necessary to stabilize the imprinted conformation of the modified enzyme ***(10,11)***, and similar results were also found during synthesis of trypsin receptors prepared by affinity-imprinting procedure ***(12,13)***. After CLIP-GO is washed it is applied to oxidized galactose (an induced substrate), which yields galactono-1,4-lactone. This product cannot be synthesized using either native GO or galactose oxidase, the well known oxidase for galactose. The product of galactose oxidase is galactonohexodialdose. Thus, following this simple biochemical modification of crude GO on a mature protein level we not only broaden the substrate spectrum of the resulting enzyme in pure aqueous solution but yield a new product not possible to achieve by any of the oxidase enzymes so far reported in the literature.

2. Materials

All chemicals used in this work are of analytical grade.

2.1. Derivatization of Glucose Oxidase

1. GO from recombinant *Aspergillus niger* (specific activity 8500 nkat mg/protein (Roche, Mannheim, Germany).
2. Itaconic anhydride (Acros Organics; www.acros.be).
3. PD-10 desalting workmate columns (Amersham Pharmacia Biotech, Uppsala, Sweden).
4. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) (Sigma Chemical Company, Steinheim, Germany).
5. Working buffer: 50 m*M* K-phosphate, pH 6.0.

2.2. Imprinting of Glucose Oxidase

1. Galactose/2 *n*-propanol (Fluka, Buchs, Germany).

2.3. Cross-Linking of Derivatized Imprinted Enzyme

1. Dry cyclohexane (Fluka, Buchs, Germany).
2. Glucose monohydrate (Fluka).
3. 30% Hydrogen peroxide (v/v) (Fluka, Buchs, Germany).
4. *2,2*'-Azobis(2-methyl-propionitrile) (AIBN; Acros Organics).
5. Ethyleneglycol dimethacrylate (EGDMA; Aldrich Chemical Company, Steinheim, Germany).
6. *o*-Dianisidine dihydrochloride (Sigma Chemical Company).
7. Horseradish peroxidase (specific activity 225 units mg/protein, one unit activity corresponds to production of 1 mg purpurogallin from pyrogallol in 20 s at pH 6.0 at 25°C) (Roche, Mannheim, Germany).

2.4. Bioconversion of Glucose or Galactose

1. Galactono-1,4-lactone (Fluka).
2. Glucono-1,5-lactone (Fluka).
3. Galactonohexodialdose (Fluka).

4. 2,6-Pyridinedicarboxylic acid (Fluka).
5. Cetyltrimethylammonium bromide (Fluka).
6. Basic anion buffer: in a 100-mL capacity stoppered conical glass flask 0.334 g 2,6-pyridinedicarboxylic acid and 10 mL cetyltrimethylammonium bromide (5 m*M*) are dissolved in 80 mL distilled water. The pH of this mixture is adjusted to 12.1 using 5 *M* NaOH and the final volume of this mixture is adjusted to 100 mL with distilled water.

3. Methods

3.1. Derivatization of Glucose Oxidase

1. The acylation of GO using various amounts of itaconic anhydride in working buffer is carried out. Here the procedure for 70% derivatization is explained.
2. In 25-mL capacity glass beaker placed on a magnetic stirrer add 60 mg of GO lyophilized powder and dissolve in 10 mL of working buffer. When the enzyme is completely soluble place this beaker in an ice bath at 4°C. A pH meter is dipped into this solution for monitoring pH during derivatization.
3. Then, slowly portion-wise, 420 mg itaconic anhydride is added with the help of a spatula (*see* **Note 1**) under constant stirring speed of 200 rpm. During this addition, pH of this solution is maintained at 6.0 using 3 *M* NaOH.
4. When all the itaconic anhydride has been added, the reaction mixture is stirred continually 30 min more in the same ice bath.
5. The unreacted monomer and other low-molecular-weight impurities ($M_r < 1000$) can be removed by gel filtration (PD 10 column) using deionized water as an eluting solvent (*see* **Note 2**).
6. All eluates are pulled together in a glass beaker at 4°C and derivatized enzyme is lyophilized to dryness and stored in a dry box at 4°C until further use (*see* **Note 3**).

3.2. Imprinting of Glucose Oxidase

1. In 10-mL capacity cellstar polypropylene tube with stopper, dissolve 30 mg of dry derivatized enzyme and 54 mg of galactose (as an imprint molecule) in 1 mLof 10 m*M* K-phosphate buffer, pH 5.0. Incubate at 25°C for 30 min.
2. GO-galactose complex is then precipitated by addition of 4 mL of supercooled (–20°C) *n*-propanol in this mixture (*see* **Note 4**). The resulting precipitated enzyme is incubated on ice for 10 min more.
3. The precipitate is collected by centrifugation at 11,000 rpm for 15 min at 4°C (Eppendorf centrifuge 5804R with F-34-6-38 rotor).
4. The pellet is washed with 1 mL of supercooled (–20°C) *n*-propanol and dried over molecular vacuum pump (Alcatel, Drytel 31) for 12 h and kept under same vacuum until further use (*see* **Note 5**).

3.3. Cross-Linking of Derivatized Imprinted Enzyme

1. In 1.5-mL capacity Eppendorf tube 10 mg of dry derivatized GO is suspended in 1 mL of dry cyclohexane using an ultrasonication bath (Branson 2200; *see* **Note 6**). The bath is filled with cold water to absorb the heat generated during sonication. This procedure requires approx 30 min.

2. To this suspended enzyme 4 mg AIBN and 200 µL EGDMA are added. Check that AIBN is completely dissolved.
3. The free radical polymerization initiated under ultraviolet irradiation (Fluotest® Forte, Atlas Materials Testing, info@atlas.com) at λ = 365 nm. The polymerization continued for 5 h at 25°C (*see* **Note 7**).
4. Keep the resulting white polymer refrigerated at 5°C for 12 h.
5. The polymer is then washed as follows: first wash with 2 mL cyclohexane to remove unreacted cross-linked, followed by three washes with 10 mL each of working buffer.
6. The protein and enzyme activity (*see* **Note 8**) are checked in aqueous washings to find out the leakage of the enzyme after cross-linking (*see* **Note 9**).
7. Cross-linked polymer is dried on a molecular vacuum pump. Similarly, a control polymer with nonimprinted, derivatized enzyme is also cross-linked in the same fashion (this is a classical immobilized enzyme).

3.4 Bioconversion of Glucose or Galactose

1. In a 2-mL capacity Eppendorf tube either 39.6 mg glucose monohydrate or 36.0 mg galactose are dissolved in 1 mL of working buffer.
2. To it either 50 mg/mL native GO or immobilized/CLIP enzyme with equivalent amount of protein (~20 mg/ mL polymer) is added.
3. This reaction mixture is saturated with air and placed in thermomixer comfort (Eppendorf) kept at 25°C with stirring speed of 1000 rpm.
4. The enzymatic reaction is terminated by heat step (i.e., increasing the temperature to 70°C after 1 h).
5. The reaction mixture is centrifuged (Eppendorf centrifuge 5417 R) at 13,000 rpm for 10 min at 4°C.
6. The supernatant is analyzed by high-performance capillary electrophoresis (HPCE; *see* **Note 10**) and high-performance liquid chromatography (HPLC; *see* **Note 11**) separately. Structure of respective products are confirmed by ^{1}H or ^{13}C NMR spectroscopy (*see* **Note 12**).
7 The bioconversion can be scaled up to a 50-mL reaction volume. For this, a stirred glass reactor (total capacity 200 mL) with continuous aeration is used. The corresponding amounts of enzyme and substrate equivalent to 50 mL reaction volume are taken in this reactor. The reaction is continued in a similar way as mentioned above except stirring at 1000 rpm is done with a mechanical propeller shaft and the temperature is maintained at 25°C by a thermostated water jacket. The products are analyzed by the same methods.

4. Notes

1. The portion-wise addition of itaconic anhydride and monitoring pH at 6.0 are the factors critical to achieving 70% derivatization.
2. The gel filtration should be carried out in a cold room.
3. The degree of derivatization is measured using TNBS assay. The detailed procedure is given in **ref. 9**. At 70% derivatization of GO an 8% increase in the specific activity is observed as compared with native enzyme.

4. The precipitant should be supercooled continuously at –20°C. The precipitation should be done abruptly in few seconds.
5. The precipitated imprinted complex is highly hygroscopic and therefore should be stored under dry conditions. The presence of traces of water in the precipitate will disrupt the imprinting efficiency.
6. Cyclohexane is dried by refluxing over metallic sodium for 12 h and then stored over molecular sieves (4 A, bead diameter 2 mm; Merck).
7. Proper lead shields should be used to work under ultraviolet radiation as the rays are mutagenic. The Eppendorf tube is placed in decline position and rotated intermittently so as to cover its maximum surface under the ultraviolet rays. Maintain the temperature of eppendorf tube strictly at 25°C during polymerization.
8. The protein is measured by Bradford method and enzyme activity is measured according to a procedure reported in **ref. *14***.
9. No enzyme leakage is found in washings after cross-linking procedure. A control polymer is synthesized for comparison with CLIP polymer. This whole process of CLIP preparation requires not more than 2.5 d.
10. HPCE is carried out using HP^{3D} capillary electrophoresis system from Agilent Technologies GmbH (Waldbronn, Germany). The system comprises a CE unit with a built-in diode-array detector and an HP^{3D} CE chemstation for system control, data collection, and analysis. Capillary electrophoresis is performed using a fused silica capillary (80.5-cm total length, 72-cm effective length, and internal diameter of 50 μm). The sample is injected with a pressure of 50 mbar for 6 s. The applied voltage is set at –15 kV and the capillary temperature is maintained at 15°C. The elution is carried out using basic anion buffer and detection is carried out using diode-array detector. The signal wavelength is set at 350 nm with a reference at 275 nm. The calibration is done using pure external standards ***(15)***.
11. The conversion of respective monosaccharide is also analyzed using HPLC. The analysis is performed on Beckman HPLC system gold, which is composed of an injection module and an automatic sampling system (Bio-Rad AS-100 HRLC) connected to a refractive index (RI) detector (Erma RI ERC-7512). The column used for detection is carbohydrate Ca^{2+} column (capillary length 300 mm and thickness 8 mm). This column is pre-equilibrated at 85°C for 30 min. Then, 50 μL appropriately diluted reaction mixture is injected into the column and product eluted using deionized water at a flow rate of 0.6 mL/min. The detection is done at 50°C using a RI detector. The calibration is performed using pure external standards. The semipreparative HPLC method is applied to isolate product from scaled-up reaction volume of 50 mL. The LiChrospher®-NH_2 column (250 × 25 mm, particle size 5 μm) is employed. The undiluted reaction mixture (3-mL portions) is injected using BioCAD® sprint system. The elution is carried out with acetonitrile:water (80:20 v/v) mobile phase at a flow rate of 39 mL/min. The fractions are collected using autofractional collector (Advantec SF-2120) and lyophilized.
12. The structure of individual product is confirmed by ^{1}H and ^{13}C NMR analysis recorded at 300 MHz with a Varian, Unity Inova™ 300-MHz instrument. The

NMR spectral data is in accordance with the proposed structures of galactono-1,4-lactone, glucono-1,5-lactone and galactonohexodialdose, respectively ***(16)***.

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17

Reversible Covalent Immobilization of Enzymes Via Their Thiol Groups

Francisco Batista-Viera, Karen Ovsejevi, and Carmen Manta

Summary

This enzyme immobilization approach involves the formation of disulfide (S–S) bonds with the support. Thus, enzymes bearing exposed nonessential thiol (SH) groups can be immobilized onto thiol-reactive supports provided with reactive disulfides or disulfide oxides under mild conditions. The great potential advantage of this approach is the reversibility of the bonds formed between the activated solid phase and the thiol-enzyme, because the bound protein can be released with an excess of a low-molecular-weight thiol (e.g., dithiothreitol [DTT]). This is of particular interest when the enzyme degrades much faster than the adsorbent, which can be reloaded afterwards. The possibility of reusing the polymeric support after inactivation of the enzyme may be of interest for the practical use of immobilized enzymes in large-scale processes in industry, where their use has often been hampered by the high cost of the support material. Disulfide oxides (thiolsulfinate or thiolsulfonate groups) can be introduced onto a wide variety of support materials with different degrees of porosity and with different mechanical resistances. Procedures are given for the preparation of thiol-reactive solid phases and the covalent attachment of thiol-enzymes to the support material via disulfide bonds. The possibility of reusing the polymeric support is also shown.

Key Words: Thiol-enzymes; enzyme thiolation; reversible enzyme immobilization; thiol-reactive supports; pyridyldisulfide-agarose; solid-phase disulfide oxides; thiolsulfinate-agarose; thiolsulfonate-agarose; β-galactosidase.

1. Introduction

Immobilization methods based on thiol-disulfide exchange reactions are unique since they allow the formation of a stable and reversible covalent bond of disulfide (S–S) type ***(1)***. Thus, enzymes bearing exposed nonessential thiol (SH) groups can

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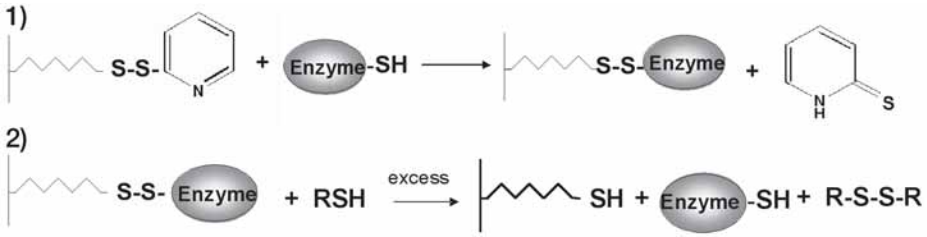

Fig. 1. Reversible covalent immobilization of thiol-enzymes onto 2-pyridyldisulfide-agarose. (1) Enzyme coupling with liberation of 2-thiopyridone, (2) elution of gel-bound enzyme with an excess of a low molecular-weight thiol (e.g., DTT or β-mercaptoethanol).

be immobilized onto thiol-reactive supports under mild conditions (e.g., low-ionic-strength buffer with pH 7.0–8.0 at room temperature). However, the applicability of these methods is not restricted to those thiol enzymes. Enzymes containing masked or unreactive thiol groups, or not containing thiol groups at all, can be modified chemically or by genetic engineering techniques, in order to provide them with reactive SH groups.

The great potential advantage of this approach is the reversibility of the bonds formed between the activated solid phase and the thiol-enzyme, because the bound protein can be released by the reduction of the disulfide bonds with an excess of a low-molecular-weight (MW) thiol (e.g., β-mercaptoethanol or dithiothreitol [DTT]). This is of particular interest when the enzyme degrades much faster than the adsorbent, which can be reloaded afterwards.

This chapter focuses exclusively on enzyme immobilization onto thiol-reactive solid phases provided with reactive disulfides or disulfide oxides. In the most traditional method, 2-pyridyldisulfide-agarose (so called PyS_2-gel), reacts with thiol groups in proteins, forming a gel-bound mixed disulfide with the protein, with concomitant liberation of 2-thiopyridone (*see* **Fig. 1**). The coupling reaction is driven essentially to completion because of the formation of the thione, a compound stabilized by thiol–thione tautomerism. The release of 2-thiopyridone (the leaving group) can be monitored in order to follow the advance of the reaction of the activated solid phase with thiols. However, its release contaminates the nonimmobilized material, which sometimes can be of interest. The use of 2-pyridyldisulfide as a ligand is very advantageous because it is reactive in a wide pH range.

2-Pyridyldisulfide-agarose has been used both for enzyme immobilization and for purification of thiol-proteins by covalent chromatography ***(1)***. Thus, crude Jack bean meal urease (a thiol-rich multimeric protein containing several nonessential thiol groups) has been reversibly immobilized onto PyS_2-gel by thiol-disulfide exchange with concomitant purification ***(2)***. A column packed with this immobilized urease derivative could hydrolyze urea very efficiently when a solution of the substrate was passed through it. Because the gel-bound active enzyme could be eluted quantitatively after incubation with low MW thiols (*see* **Fig. 1**), the method was also useful for the purification of urease (e.g., 167-fold) by covalent chromatography.

Pancreatic hog α-amylase (containing thiol groups required for high activity but unreactive towards pyridyldisulfide-agarose) was provided with "*de novo*" thiol groups through a mild thiolation process, allowing its immobilization onto PyS_2-gel with high yields ***(3)***. The immobilized α-amylase derivative was used in a packed-bed reactor for the continuous hydrolysis of starch; when the enzymatically active gel had lost its activity, it could be regenerated *in situ* by reductive uncoupling of the inactive protein (*see* **Fig. 1**) and attachment of a new portion of thiolated α-amylase.

A disadvantage of these gels is that at high ionic strength (e.g., 0.5 *M* sodium or potassium sulfate) 2-pyridyldisulfide-gels bind some proteins lacking thiol groups, especially immunoglobulins, through a noncovalent interaction, a property that has been utilized in the so-called thiophilic adsorption chromatography ***(4)***.

Thiol-reactive adsorbents based on pyridyldisulfide groups are commercially available. Thus, Amersham Biosciences (Uppsala, Sweden), provides two types of such agarose derivatives which differ both in the length of the spacer arm and the degree of substitution. Activated Thiol Sepharose 4B contains about 1 μmol pyridyldisulfide groups per milliliter packed gel, and a glutathione (10 atoms) spacer arm. Thiopropyl Sepharose 6B contains about 20 μmol pyridyldisulfide groups per milliliter packed gel, and a 2-hydroxypropyl (4 atoms) spacer arm. It should be borne in mind that in spite of its trade name, Thiopropyl Sepharose 6B is not a thiol-gel but a mixed reactive disulfide gel.

More recently, a new approach based on agarose-bound disulfide oxides groups (thiolsulfinates and thiolsulfonates) has been developed for the reversible immobilization of thiol-containing substances ***(5)***. These agarose-derivatives display high reactivity and selectivity towards both low- and high-MW thiols and can be used for the reversible immobilization of thiol–peptides and thiol–proteins ***(5–8)***. Enzymes containing exposed SH groups are covalently immobilized onto solid-phase disulfide oxides under mild conditions. The immobilization process involves the formation of disulfide bonds between thiol groups on the enzyme and disulfide oxide structures on the supports.

Because of displacement of the electrons around the two sulfur atoms, disulfide oxides show high *S* reactivity. Thiol-containing molecules react with the more electrophilic of the two sulfur atoms (the unoxidized one) and become, as a result, immobilized to the solid phase by disulfide bonds (*see* **Figs. 2** and **3**). Contrary to the case with 2-pyridyldisulfide-based gels, the leaving groups (sulfenic or sulfinic acid) remain attached to the support.

All the techniques for introducing either reactive disulfides of 2-pyridyldisulfide type or disulfide oxides (thiolsulfonates or thiolsulfinates) into beaded agarose gels start with the same support derivative: thiol-agarose, which is prepared by means of a three-step thiolation process. Thus, to obtain a thiol-substituted agarose, the support is first reacted with epichlorohydrin in a strong alkaline medium to introduce oxirane moieties into the gel; the oxirane groups are then converted with sodium thiosulfate to gel-bound thiosulfate groups (Bunte salt derivative), which finally are reduced with DTT to thiol groups (*see* **Fig. 4**). The degree of substitution in the thiol-agarose can be regulated by the amount of epichlorohydrin added as well as by the incubation conditions (e.g., incubation period, temperature) ***(9)***.

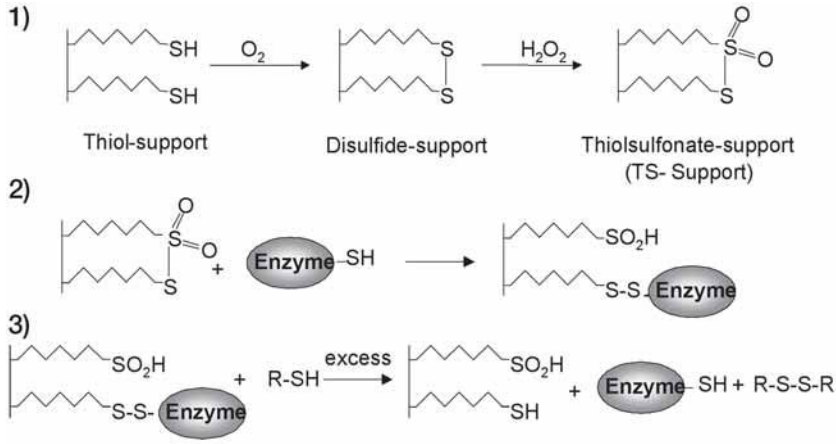

Fig. 2. Reversible covalent immobilization of thiol-enzymes onto a thiolsulfonate-support. (1) Preparation of a thiolsulfonate-support through oxidation of thiol-support. (2) Immobilization of a thiol-enzyme onto a thiolsulfonate-support; the leaving group (sulfinic acid group) remains attached to the support. (3) Release of gel-bound enzyme with an excess of a low-molecular weight thiol.

Thiol-agarose can be subsequently converted to thiolsulfonate-agarose (TS-gel) or thiolsulfinate-agarose (TSI-gel) through different oxidation procedures. Thus, oxidation of thiol-agarose with hydrogen peroxide at moderately acidic pH and room temperature for extended periods (20–30 h) converts thiol groups on the support (via disulfide and thiolsulfinate) into thiolsulfonate (disulfide dioxide) moieties (*see* **Fig. 2**) ***(6)***. The thiolsulfinate (disulfide monoxide) groups are introduced by oxidation of thiol-agarose. The method comprises two steps: first, mild oxidation of agarose-bound thiol groups to disulfide structures with potassium ferricyanide; second, controlled oxidation of the agarose disulfide groups so formed to thiolsulfinate groups is performed with the oxidizing agent magnesium monoperoxyphthalate (*see* **Fig. 3**) ***(7,8)***. This reagent makes possible the introduction of only one oxygen atom per immobilized aliphatic disulfide group to form a thiolsulfinate moiety. The number of thiolsulfinate groups introduced can be regulated at will by choosing a certain molar ratio between the oxidizing agent and the gel-bound disulfide structures. When the stoichiometric quantity of monoperoxyphthalate is used, maximum thiol-binding capacity is achieved; if half of this amount is used, 50% of the maximum thiol-binding capacity is obtained, and so on.

Thus, it is possible to prepare thiolsulfinate-agarose gels with different thiol-binding capacities from the same thiol-agarose batch.

The gel-bound thiolsulfinate/thiolsulfonate groups are very stable in the pH range of 3.0 to 8.0; therefore, solid phases containing these groups can be stored as suspensions at pH 5.0 at 4°C for extended periods, without a decrease in their thiol-binding capacity. Furthermore, disulfide oxide gels do not need any preservative agent since no bacterial or fungal growth was observed after storing at 4°C at pH 5.0 for 2 yr.

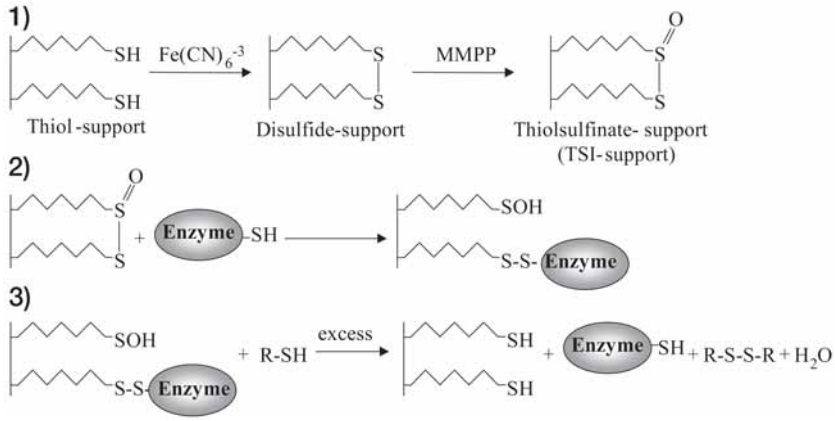

Fig. 3. Reversible covalent immobilization of thiol-enzymes onto a thiolsulfinate-support. (1) Preparation of a thiolsulfinate-support through oxidation of thiol-support. (2) Immobilization of a thiol-enzyme onto a thiolsulfinate-support; the leaving group (sulfenic acid group) remains attached to the support. (3) Release of gel-bound enzyme with an excess of a low-molecular weight thiol.

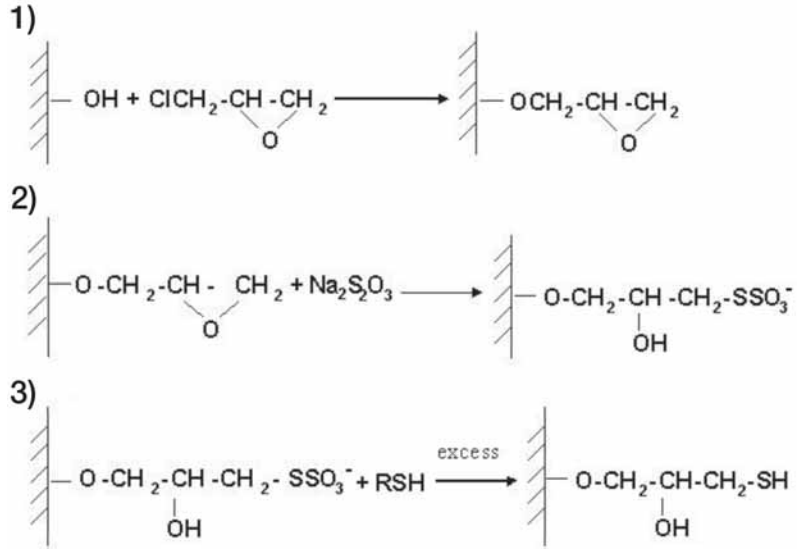

Fig. 4. Synthesis of a thiol-support through a three-step procedure. (1) Epoxy-activation of the solid phase. (2) Formation of thiosulfate-ester estructures (Bunte salt derivative). (3) Reduction of the Bunte salt by an excess of a low-molecular-weight thiol.

After extensive reuse of an enzyme derivative until its inactivation, the disulfide bonds can be split under reducing conditions at alkaline pH to remove the bound protein (*see* **Figs. 2** and **3**). Then, the gel can be regenerated following the activation techniques described above and reloaded with fresh enzyme. This is achieved more efficiently with a TSI-gel, resulting from the fact that the sulfenic acid groups (SOH) formed during thiol coupling can easily be converted back to SH moieties by using an excess of a mild reducing agent (*see* **Fig. 3**). Thus thiolsulfinate-agarose can, at least in theory (as is also the case with 2-pyridyldisulfide-agarose), be regenerated an unlimited number of times. In contrast, TS-gels can be regenerated only a few times, owing to the formation of gel-bound nonreducible sulfinate groups (*see* **Fig. 2**), with a concomitant decrease of about 50% of its thiol-binding capacity after each cycle ***(7)***.

Besides beaded agarose, thiolsulfonate and thiolsulfinate groups can be introduced onto a wide variety of support materials with different degrees of porosity and with different mechanical resistances (e.g., soft particles such as Sephadex and cellulose; semirigid ones such as Toyopearl® resins, Sephacryl™, Eupergit®, Superdex™, and Superose™; and rigid particles such as controlled porous glass). Thiolation of the different supports is carried out using the same procedure as reported for agarose, except for porous glass which is directly thiolated by silanization in organic solvent with 3-mercaptopropyltriethoxysilane, and for oxirane-carrying acrylic beads (Eupergit C, Sepabeads® EP), for which the first step in the thiolation procedure is omitted ***(8)***.

Table 1 shows thiol-binding capacities of different thiolsulfinate-supports prepared according to the methods described. These figures represent the total number of thiol-reactive structures, because they are determined by measuring the maximum binding of a thiol-peptide (reduced glutathione). In spite of the disparate types of support assayed, it is possible to provide all of them with thiol-reactive groups.

In some cases the degrees of activation achieved were lower than expected, as a consequence of the rigidity of some matrices that makes impossible the formation of disulfide bonds between SH groups located far from each other.

The application of TS- and TSI-gels to the immobilization of high-MW thiols was assessed using different enzymes: with exposed nonessential SH groups, with buried thiol groups, with *de novo* thiol groups, and with reducible disulfides. Thus, immobilization of β-galactosidase (*Escherichia coli*) on thiolsulfonate- and thiolsulfinate-agarose has been performed with yields higher than 80% and a high percentage of expressed activity ***(7,10)***. The maximum capacity of these thiol-reactive adsorbents is on the order of 10 mg protein/mL. In the case of commercial β-galactosidases, such as those from *Kluyveromyces lactis* and *Aspergillus oryzae*, a previous reduction process was shown to be essential in order to unblock their more exposed thiol groups ***(11)***. This can be done either with soluble reducing agents such as DTT or more conveniently with solid-phase reducing agents ***(12)***. When enzymes lack reactive SH groups, one alternative can be the introduction of *de novo* thiol groups by a thiolation process using suitable heterobifunctional reagents, as in the case of sweet potato β-amylase and pullulanase ***(13,14)***. Another possibility is site-directed mutagenesis techniques that can introduce a free cysteine at a suitable position on the protein, which can be used subsequently

Table 1
Thiol-Binding Capacities for Different Thiolsulfinate-Supports Determined by Glutathione Binding *(8)*

Support: nature and identification	SH group content of thiol-support (µmoles/g dried deriv.)	TSI-group content (µmoles/g dried deriv.)	TSI-group content (µmoles/g wet deriv.)[a]
Polysaccharides			
Sepharose 4B	1009	500	27
Cellulose	194	82	34
Sephadex G-75	397	386	38
Superose 12	414	183	22
Composite support			
Superdex 75	272	212	26
Sephacryl S-200 SF	244	217	31
TSK-gel HW-65F	355	105	20
Eupergit	313	61	15
Sepabeads	124	52	32
Inorganic supports			
Porous glass (CPG)	474	125	74

[a]Because supports have quite different swelling factors in water, it is sometimes more useful to know the binding capacity in µmol of GSH per g of swollen and suction-dried (wet) derivative.

for immobilization. Thus, genetically engineered glucose dehydrogenase has been immobilized on a Thiopropyl–Sepharose column ***(15)***.

After enzyme immobilization onto TS/TSI-gels, the remaining reactive groups in the matrix could react with hidden thiol groups in the protein, leading to an activity loss, specifically during thermal treatments. In order to avoid this effect, excess reactive structures can be blocked by coupling polar low-MW thiols. Thus, a β-galactosidase derivative blocked with glutathione retained 100% of initial activity after 1 h at 50ºC, whereas the unblocked derivative only retained 16% of the activity ***(10)***.

On the other hand, different nanoenvironments can be generated by reacting the excess of gel-bound disulfide oxides moieties with apolar, polar, anionic, or cationic thiols. In that way, it might be possible to tailor the matrix surface so as to maximize enzyme stability in particular applications ***(10,16)***.

In conclusion, reversible covalent immobilization of enzymes through thiol-disulfide exchange reactions has the following advantages.

- It involves thiol groups, which are generally the most reactive groups found in proteins, because of the high nucleophilicity of the corresponding thiolate ions.
- Because thiolate ions exist at reasonable concentrations at neutral to weakly alkaline pH values, it is possible to perform enzyme immobilization under mild conditions and at relative high rates.

- These immobilization methods are absolutely specific for thiols.
- Resulting from the fact that thiol groups are scarce in proteins and located in specific regions, the enzyme can be immobilized in an oriented way, exposing the active site.
- Immobilization methods based on thiol-disulfide exchange reactions are unique, because they combine a very stable covalent attachment with the possibility of releasing the bound material. Thus, after enzyme inactivation, the immobilized material can be easily released by reduction.
- Disulfide oxides can be introduced onto a wide variety of support materials with different degrees of porosity and with different mechanical resistances that make them suitable for many analytical and preparative applications.
- The activated supports show high storage stability because thiol-reactive groups remain unchanged after 2 yr at 4°C.
- Sulfur content makes these gels highly resistant to microbial growth, so it is not necessary to use sodium azide as a preservative.
- The activated gel structures are completely regenerable for TSI- and PyS2-gels, and 50% regenerable for TS-gels.
- The possibility of reusing the polymeric support after inactivation of the enzyme may be of interest for the practical use of immobilized enzymes in large-scale processes in industry, where their use has often been hampered by the high cost of the support material.

In **Subheading 3.**, procedures are given for the preparation of thiol-reactive solid phases and the covalent attachment of thiol-enzymes to the support material via disulfide bonds. The possibility of reusing the polymeric support is also shown after reductive detachment, chemical reactivation, and reattachment of fresh thiol protein.

2. Materials

2.1. Preparation of Activated Solid Phases

2.1.1. Preparation of Thiol-Agarose and Titration of Thiol Groups

1. Sepharose 4B (Pharmacia BTG, Uppsala, Sweden).
2. Epichlorohydrin (1-chloro-2,3-epoxypropane) (Sigma).
3. Sodium thiosulfate (Fluka AG, Buchs, Switzerland).
4. Dithiothreitol (DTT) (Sigma).
5. 2,2'-Dipyridyldisulfide (2-PDS) (Sigma).

2.1.2. Preparation of 2-Pyridyldisulfide Agarose and Titration of Pyridyl Disulfide Groups

1. Mercapto pyridine (Fluka AG, Buchs, Switzerland).
2. 2,2'-Dipyridyldisulfide (2-PDS) (Sigma).
3. DTT (Sigma).

2.1.3. Preparation of Thiolsulfonate-Agarose (TS-gel)

1. Perhydrol® (30% hydrogen peroxide) (Merck AG, Darmstadt, Germany).

2.1.4. Preparation of Thiolsulfinate-Agarose (TSI-gel).

1. Magnesium monoperoxyphthalate (MMPP, Merck AG).
2. Potassium ferricyanide (Baker, Phillipsburg, NJ).

2.1.5. Titration of Thiol-Reactive Structures in the Agarose Derivatives

1. Reduced glutathione (Sigma).
2. 2,2'-Dipyridyldisulfide (2-PDS, Sigma).

2.2. Immobilization of E. coli β*-Galactosidase Onto Thiol-Reactive Agarose*

For each of the following techniques it is necessary to have:

1. Empty PD-10 columns.
2. End-over-end mixer.
3. Vacuum pump.

2.2.1. Activity Determination

1. Activity buffer: 0.1 *M* potassium phosphate buffer, pH 7.5, containing 3 m*M* magnesium chloride.
2. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG, Sigma).

2.2.2. Immobilization of the Enzyme

1. Thiolsulfonate-agarose, prepared as described in **Subheading 3.1.3**.
2. Immobilization buffer: 0.1 *M* potassium phosphate buffer, pH 7.0.
3. 0.1 *M* potassium phosphate buffer, pH 7.0, 0.5 *M* NaCl .
4. β-Galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) grade VIII from *E.coli*. (Sigma).

2.2.3. Blocking Excess of Active Groups

1. Glutathione (GSH, Sigma).

2.2.4. Enzyme Elution

1. DTT (Sigma)

2.3. Immobilization of K. lactis β*-galactosidase Onto Thiol-Reactive Supports*

2.3.1. Activity Determination

1. Activity buffer: 20 m*M* potassium phosphate buffer, pH 7.0, containing 2 m*M* magnesium chloride, 0.1 *M*, KCl.
2. ONPG (Sigma).

2.3.2. Protein Determination

1. Coomassie Plus protein assay reagent (Pierce, Rockford, IL).

2.3.3. Immobilization of the Enzyme

1. Thiolsulfonate-agarose (TS-gel) and thiolsulfinate-agarose (TSI-gel) prepared as described in **Subheadings 3.1.3.** and **3.1.4.** respectively.

2. Immobilization buffer: 0.1 *M* potassium phosphate buffer, pH 7.0.
3. 0.1 *M* potassium phosphate buffer, pH 7.0, 0.5 *M* NaCl .
4. β-Galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) from Gist-Brocades (Gif-sur-Yvette, France).
5. DTT (Sigma).

2.3.4. Blocking Excess of Active Groups

1. GSH (Sigma)

2.3.5. Enzyme Elution

1. DTT (Sigma).

3. Methods

3.1. Preparation of Activated Solid Phases

3.1.1. Preparation of Thiol-Agarose

The preparation of mercaptohydroxypropyl ether agarose gel (thiol-agarose) is carried out essentially as described by Axén et al. ***(9)***. In this method the agarose beads (Sepharose 4B) are first reacted with epichlorohydrin in an alkaline medium. The oxirane groups thus formed are then converted with sodium thiosulfate to gel-bound thiosulfate groups (Bunte-salt), which are finally reduced with DTT to thiol groups (*see* **Fig. 4**).

1. Suspend 15 g suction-dried Sepharose 4B in 15 mL of 1 *M* NaOH.
2. Add slowly with agitation 2.5 mL epichlorhoydrin (*see* **Note 1**).
3. Incubate overnight at 22°C under shaking (*see* **Note 2**).
4. Transfer the epoxy-activated gel to a sintered-glass filter and wash it with distilled water. Use it immediately.
5. Equilibrate the gel with 0.5 *M* sodium phosphate buffer, pH 6.3, and suspend it in 15 mL of the same buffer.
6. Add 15 mL 2 *M* sodium thiosulfate and incubate overnight under shaking at 22°C (minimum 6 h).
7. Transfer the Bunte-salt gel to a sintered-glass filter and wash it with water. Store it in distilled water at 4°C until use (very stable) (*see* **Note 3**).
8. Suspend 15 g suction-dried Bunte-salt gel with 15 mL 0.2 *M* sodium bicarbonate buffer, pH 8.5.
9. Dissolve 3 g DTT in 15 mL 1 m*M* ethylene diamine tetraacetic acid (EDTA) and add it to the Bunte-salt gel suspension (*see* **Note 4**).
10. Incubate the mixture during 1 h under shaking at 22°C.
11. Transfer to a sintered-glass filter and wash with:
    a. 0.2 *M* sodium bicarbonate buffer, pH 8.5.
    b. Distilled water.
    c. 0.1 *M* acetic acid, until absence of DTT.

These conditions will give a thiol-agarose derivative containing between 400 and 600 μmoles of thiol groups per g of dried gel (*see* **Note 5**).

3.1.2. Thiol Group Analysis

1. The thiol content of both soluble and insoluble material is determined spectrophotometrically by titration with 2,2'-dipyridyldisulfide (saturated solution, 1.5 m*M*, prepared by 30 min agitation followed by filtration) dissolved in 0.1 *M* sodium phosphate, pH 8.0, according to Brocklehurst et al. ***(17)***.

3.1.2.1. Preparation of 2-Pyridyldisulfide-Agarose

The preparation of 2-pyridyldisulfide-agarose is carried out as reported by Oscarsson et al. ***(4)***.

1. Suspend 10 g of suction-dried thiol-agarose gel in 40 mL of 50 m*M* sodium phosphate buffer, pH 8.5.
2. Dissolve 1.8 g of 2-mercaptopyridine and 3.5 g of 2,2'-dipyridyldisulfide in 40 mL of 98% ethanol.
3. Add this solution to the gel suspension and stir for 15 h at room temperature.
4. Wash with ethanol and water on a glass filter.

3.1.2.2. Titration of Pyridyldisulfide Groups

The determination is based on the release of 2-thiopyridone after reduction of 2-pyridyldisulfide-gel. The extinction coefficient at 343 nm for 2-thiopyridone is $8.02 \times 10^3\ M^{-1}\ cm^{-1}$.

1. A filter-dried gel aliquot is weighed and suspended in 25 m*M* DTT in 0.1 *M* phosphate buffer, pH 8.0.
2. After incubation under shaking at room temperature for 30 min, the supernatant is filtered and the absorbance at 343 nm is measured.

3.1.3. Preparation of Thiolsulfonate-Agarose (TS-Gel) **(5,6)**

1. Suction-dried thiol-agarose (15 g containing 500–800 µmoles SH groups/g dried gel) is suspended in 45 mL of 0.2 *M* sodium acetate, pH 5.0.
2. Add aliquots of 30% hydrogen peroxide under continuous shaking, 1.8 mL initially and then 2.2 mL after 30, 90, and 150 min. The incubation is then continued to give a total reaction time of 30 h (*see* **Note 6**).
3. Transfer the oxidized gel to a sintered-glass filter and wash with 0.1 *M* acetic acid until free of hydrogen peroxide.
4. Store the activated gel in 0.2 *M* sodium acetate, pH 5.0, at 4°C until use.
5. The thiolsulfonate group content of the gel thus obtained is between 250 to 400 µmoles TS groups per g of dried gel.

3.1.4. Preparation of Thiolsulfinate-Agarose (TSI-Gel) in Two Steps **(7,8)**

3.1.4.1. Disulfide-Agarose (S_2-Gel)

1. Suspend fifteen grams of suction-dried thiol-agarose gel in 30 mL of 0.1 *M* sodium phosphate buffer, pH 7.0, and add 0.1 *M* potassium ferricyanide (by 0.5-mL aliquots under shaking until the yellow color persists for at least 30 min).
2. Then wash the gel thoroughly on a sintered-glass filter with buffer, 1 *M* NaCl, and 0.2 *M* sodium acetate buffer, pH 5.0. It can be assumed that the disulfide (S–S) group content of obtained S_2-gel is 50% of the thiol content in starting thiol-agarose.

3.1.4.2. Thiolsulfinate-Agarose (TSI-Gel)

1. Suspend 15 g of suction-dried S_2-gel in 100 mL 0.2 *M* sodium acetate, pH 5.0, in which the required amount of magnesium monoperoxyphthalate has been dissolved (0.5 moles per mol of S–S groups) (*see* **Note 7**). The suspension is incubated while shaking for 2 h at room temperature (22°C).
2. Then thoroughly wash the gel derivative on a sintered-glass filter with 50 m*M* sodium acetate buffer, pH 5.0, and 0.1 *M* acetic acid.
3. The washed gel is stored at 4°C as a suspension in 0.2 *M* sodium acetate buffer, pH 5.0 (*see* **Notes 8** and **9**).

3.1.5. Titration of Thiol-Reactive Structures in the Agarose Derivatives

This is performed by back titration of remaining GSH free in solution, after its incubation with thiol-reactive gels. A blank is performed for spontaneous oxidation of GSH.

1. Equilibrate 2.0 g suction-dried gel aliquots (TS- or TSI-gel) with 0.1 *M* sodium phosphate buffer, pH 7.0, in centrifuge tubes.
2. Adjust the amount in each tube to 3.0 g with the same phosphate buffer .
3. Add 3-mL aliquots of 15 m*M* glutathione dissolved in the same buffer to each tube while mixing (Vortex).
4. Incubate the suspensions for 30 min at 22°C , mix every 5 min, and then centrifuge.
5. Mix 50-µL aliquots of supernatants with 3.0 mL of 1.5 m*M* 2,2'-dipyridyldisulfide dissolved in 0.1 *M* sodium phosphate buffer, pH 8.0.
6. Measure the absorbance at 343 nm.

3.2. Immobilization of E. coli β*-Galactosidase Onto Thiol-Reactive Agarose* (10)

3.2.1. Activity Determination

1. The free β-galactosidase activity is determined using 14 m*M* ONPG as a substrate in 0.1 *M* potassium phosphate buffer, pH 7.5, containing 3 m*M* magnesium chloride (activity buffer) (*see* **Note 10**). The released *o*-nitrophenol is determined spectrophotometrically at 405 nm ***(18)***.
2. The immobilized enzyme activity is assayed by incubating 100 µL aliquots of gel suspensions (containing 10 mg of suction-dried gel derivatives) with ONPG in activity buffer, using a 1 cm path length cuvet provided with magnetic stirring.

3.2.2. Protein Determination

1. The protein concentration is estimated from the absorbance values at 280 nm and using an extinction coefficient of 2.09 for a 1 mg/mL solution of *E. coli* β-galactosidase ***(18)***.
2. The protein content of the gel derivatives is determined by total amino acid analysis, after extensive drying in a dessicator over phosphorus pentoxide and hydrolysis in 6 *M* HCl for 24 h at 110°C.

3.2.3. Preparation of Immobilized Enzyme Derivatives

1. Wash thiolsulfonate-agarose (TS-gel) in a sintered-glass filter connected to a vacuum pump, with 0.1 *M* potassium phosphate buffer, pH 7.0 (immobilization buffer) (*see* **Note 11**).
2. Transfer 2-g aliquots of suction-dried TS-gel equilibrated in immobilization buffer to empty PD-10 columns (*see* **Note 12**).
3. Add 7.5 mL of β-galactosidase solutions (containing between 1.0 and 50.0 mg of protein, respectively) in 0.1 *M* potassium phosphate buffer, pH 7.0 (*see* **Note 13**).
4. Mix the suspensions gently in an end-over-end mixer for 16 h at 4°C.
5. Wash the resulting insoluble derivatives sequentially with 25-mL portions of 0.1 *M* potassium phosphate buffer, pH 7.0, with and without 0.5 *M* NaCl (*see* **Note 14**).
6. Equilibrate and dilute the conjugates to 22 mL with 0.1 *M* potassium phosphate buffer, pH 7.5, containing 3 m*M* magnesium chloride (activity buffer).
7. Store at 4°C (*see* **Note 15**).

3.2.4. Blocking Excess Active Groups

1. Incubate for 30 min under mixing, 2.0 g of suction-dried gel derivatives with 20.0 mL of 8 m*M* glutathione solution. After incubation, filtrate, wash and dilute to 20.0 mL with activity buffer.
2. Assay β-galactosidase activity in both the filtrates and blocked-gel suspensions (*see* **Note 16**).

3.2.5. Enzyme Elution

1. Incubate aliquots of suction-dried derivatives (100 mg) with 4.0 mL of fresh 50 m*M* DTT in 0.1 *M* sodium phosphate, pH 8.5, with mixing for 1 h under end-over-end rotation.
2. After filtration, determine activity in the filtrates (*see* **Note 17**).

3.3. Immobilization of K. lactis β*-Galactosidase Onto Thiol-Reactive Supports* (11)

3.3.1. Activity Determination

The activity determination is performed as in **Subheading 3.2.1.**

3.3.2. Protein Determination

1. The protein content of the enzyme solutions is determinated with Coomassie Plus reagent.
2. Immobilized protein is estimated as the difference between the amount of protein added to the gel and that recovered in the pooled supernatant and washing fractions. It is also determined by total amino acid analysis after extensive drying over phosphorus pentoxide in a dessicator and hydrolysis in 6 *M* HCl for 24 h at 110°C.

3.3.3. Enzyme Reduction With a Soluble Reducing Agent

1. Incubate aliquots of enzyme (60 mg/mL, 1400 enzyme units [EU]/mL) at 22°C with DTT 200 m*M* dissolved in 20 m*M* potassium phosphate, pH 8.0 (*see* **Notes 18** and **19**).

2. After 30 min, remove the excess of DTT by gel-filtration.
3. Determine protein SH content ***(21)***.

3.3.4. Enzyme Reduction With a Solid-Phase Reducing Agent

Using thiol-agarose containing 1000 μmoles SH groups per gram of dried gel, prepared as described in **Subheading 3.1.1.**

1. Incubate aliquots of enzyme (8 mg/mL, 240 EU/mL) at 22°C with 1.0 g suction-dried thiopropyl-agarose.
2. After 2 h, remove the reducing agent by filtration on a sintered glass filter.
3. Titrate the thiol content of the reduced enzyme **(*21*)** (*see* **Notes 20–22**).

3.3.5. Preparation of Immobilized Enzyme Following a Batch Procedure

1. Follow the protocol described in **Subheading 3.2.3.** using reduced enzyme (*see* **Notes 23–25**).

3.3.6. Preparation of Immobilized Enzyme Following Column Procedure

1. Equilibrate TS-agarose or TSI-agarose as in **Subheading 3.2.3.**
2. Pack 5 mL of this gel into a plexiglass column.
3. Recirculate for 16 h at 22°C, reduced and gel filtered enzyme in 0.1 *M* phosphate buffer, pH 7.0, at a flow rate of 10 mL/h.
4. Wash the column sequentially with 0.1 *M* phosphate buffer, pH 7.0, with and without 0.5 *M* NaCl.
5. Measure β-galactosidase activity in the recirculated and washing fractions, and in the gel-derivative obtained (*see* **Note 26**).

3.3.7. Blocking Excess Active Groups

1. Incubate for 30 min, 300 mg of suction-dried gel derivatives with 3.0 mL of fresh 8 m*M* glutathione solution.
2. After incubation, filter, wash, and dilute to 3.0 mL with activity buffer (*see* **Note 27**).

3.3.8. Enzyme Elution

1. Follow the protocol described in **Subheading 3.2.5.**
2. After filtration, determine protein content and activity in the filtrates (*see* **Note 28**).

3.3.9. Lactose Hydrolysis in Batch

1. Incubate at 22°C and 37°C, aliquots of free and immobilized enzyme suspensions with 5% lactose saline solution, whey, whey permeates and skimmed milk (ratio 1/10 [v/v]).
2. Glucose formation is followed by an enzymatic method (*see* **Note 29**).

3.3.10. Lactose Hydrolysis in Column

1. A column with 5 mL of packed gel is fed with the same lactose solutions specified above, using a flow rate between 6.0 and 10.0 mL/h.
2. Determine the glucose formed (*see* **Note 30**).

3.3.1. Reuse of the Immobilized Enzyme

1. Incubate with whey at 22°C for 2.5 h an aliquot of the TSI-agarose-β-galactosidase derivative.
2. Determine the amount of glucose formed.
3. Wash the derivative with activity buffer.
4. Use the washed derivative for a second time in the same way as in the first time.
5. This protocol is carried out four times (*see* **Note 31**).

3.3.12. Regeneration of Thiol-Reactive Gels

After release of bound material from the gel derivative by reductive cleavage and thorough washing, reactivation is performed according to the two-step procedure described for TSI-gel synthesis or the one-step procedure described for TS-gel (*see* **Notes 32** and **33**).

4. Notes

1. According to Axén et al. ***(9)*** it is possible to regulate the degree of thiol agarose substitution (thiol content) by varying the amount of epichlorohydrin used in the epoxy activation step.
2. This reaction is highly dependent on temperature and on incubation time. It is possible to decrease incubation time by working at 60°C.
3. It is possible to confirm the highly charged properties of the Bunte-salt gel by observing the increase in the swelling and in the volume of the gel.
4. Reduction of the Bunte-salt gel is carried out by using a small excess of DTT (e.g., 10%) with respect to the stoichiometric amount. This is important because of the high cost of this reagent.
5. A higher content of thiol groups (e.g., 1000 μmoles per gram of dried gel) would be desirable if the thiol-agarose is intended for use as a solid-phase reducing agent.
6. Longer incubation periods lead to a loss of activated groups ***(6)***.
7. Magnesium monoperoxyphthalate hexahydrate (MMPP) is a cheap and safe oxidant which can be purchased from Merck Schuchardt (Darmstadt, Germany; Merck Schuchardt MS Info 88-1, cat. no. 818372). It is a crystalline solid that is soluble in water and low MW alcohols, and it was originally recommended for the selective oxidation of organic sulfides to sulfoxides and sulfones ***(19,20)***.
8. TS- and TSI-gels show high stability in 0.2 *M* sodium acetate, pH 5.0, at 4ºC, because no decrease in reactivity can be detected after long storage periods under these conditions ***(6,8)***.
9. The antibacterial properties of alkyl thiolsulfinates are well known and we have never observed microbial growth in these gel suspensions.
10. This potassium phosphate buffer is the most suitable for this β-galactosidase because sodium ions inactive the enzyme, and also because of the presence of magnesium ions, which stabilizes the enzyme.
11. Use gentle suction to remove liquid until the moment when the gel forms a firm cake and then stop suction; the gel is then called suction-dried gel.
12. Plastic reservoir with a bottom membrane and top and bottom caps.

13. This *E. coli* β-galactosidase is selected because of its high cysteine content; it should therefore be possible to use its nonessential thiol groups for its immobilization.
14. Sodium chloride is included in the washing buffer to elute nonspecifically bound proteins.
15. The percentage of gel-bound activity depends on the amount of protein added, ranging from 70% to 80% for the low- and intermediate-load derivatives, to 41% for the high-load derivative. A control gel (with its thiol-reactive groups previously blocked with glutathione) does not retain the enzyme.
16. In 3.0 mL of 8 m*M* glutathione solution there is a tenfold excess of glutathione with respect to the number of active groups present on the activated gel. Other thiol compounds, like mercaptopropionic acid and mercaptoethanol (in activity buffer) can be used for blocking the excess of active groups. Because of its low pKa value, cysteamine (pKa = 8.3) is the only thiol-compound assessed that has an eluting effect. (After the blocking treatment with cysteamine under the conditions described, nearly 30% of the bound enzyme is released.)
17. More than 90% of the total immobilized activity is eluted, proving that the matrix-enzyme bonds formed are of disulfide type.
18. Prior to its use, this commercial lactase preparation (Maxilact LX-5000) is diluted 10-fold with 20 m*M* phosphate buffer, pH 7.0, and gel filtered through Sephadex G-25.
19. Under these conditions, the ratio μmoles SH groups of reductant/mg of protein is 3.4.
20. Achieving a ratio of μmoles SH groups of reducing agent/mg of protein of 0.5.
21. The reduction of this lactase with both reductants allowed a threefold increase in its initial content of SH groups. Nearly sevenfold less μmoles of SH groups/mg of protein were needed to perform the reduction of *K. lactis* β-galactosidase with thiopropyl-agarose than the SH amount required for the reduction with DTT.
22. After the reduction process, the remaining content of SH groups of the solid-phase reducing agent was quantified; nearly 60% of the initial SH groups remained.
23. The reduction process dramatically improved the immobilization yield onto thiol-reactive supports, from 0% for the native enzyme up to 90% for the reduced enzyme.
24. Using an acrylic resin as support, like Toyopearl HW-65F, the expressed β-galactosidase activity is strongly dependent on the salt concentration present during the immobilization process, increasing from 32% in absence of salt to 60% in the presence of 0.3 *M* K_2SO_4 (**Table 2**).
25. The immobilization process requires incubation periods of at least 8 hours for quantitative binding of the reduced enzyme to TS- and TSI-supports (*see* **Fig. 5**).
26. After overnight recirculation, the immobilization yield for the reduced enzyme is 69%.
27. The blocking treatment markedly improves the thermal stability at 37°C (optimum temperature for the hydrolysis of lactose) of β-galactosidase derivatives (**Table 3**).

Table 2
Immobilization Yields of Reduced β-Galactosidase Onto Thiol-Reactive Supports

Thiol-reactive support	Immobilization yield (%)[a]	Expressed activity (%)[b]
TS-agarose	91	82
TSI-agarose	90	86
TSI-Toyopearl	74	*60*

[a]Estimated on the basis of the difference between the amount of protein applied and that remaining in supernatants and washings.

[b]Percentage of expressed activity (gel-bound activity/ applied activity).

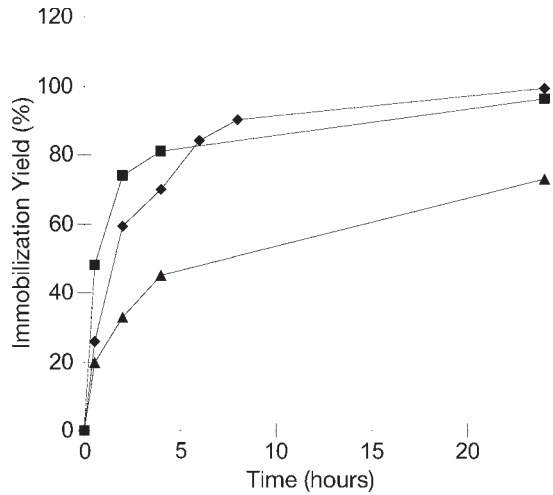

Fig. 5. Kinetics for the immobilization of reduced K. lactis β-galactosidase onto thiol-reactive supports. ◆, TS-agarose; ■,TSI-agarose; ▲, TSI-Toyopearl.

28. Nearly 70% of the total immobilized activity and 90% of the bound protein are eluted, showing the reversibility of the immobilization process.
29. The percentages of lactolysis (lactose hydrolysis) at 22°C are similar, regardless of the lactose solution (nearly 75% after 90 min of reaction).
30. When this mini-reactor is fed with whey permeate at 22°C, steady state lactose conversion reaches 90% and remains constant for 12 d. When it is fed for another 5 d with skimmed milk, the percentage of lactolysis is maintained.
31. After five cycles of 2.5 h each, the degree of lactolysis remains greater than 75%.
32. It is important to emphasize the differences found between TS- and TSI-gels from the point of view of regeneration. TSI-gels can be regenerated to 100% of their initial reactivity, whereas TS-gels can only be regenerated to 50% (**Table 4**).

Table 3
Stability of Immobilized β-Galactosidase Derivatives (Before and After Blocking With GSH) as a Function of the Incubation Period at 37°C.

Enzyme derivative	Remaining activity (%) after incubation (h) 0.5	1	2
TS-agarose-β-gal	80	60	52
Blocked TS-agarose-β-gal	100	100	92
TSI-agarose-β-gal	94	84	50
Blocked TSI-agarose-β-gal	100	100	81
TSI-Toyopearl-β-gal	76	69	55
Blocked TSI-Toyopearl-β-gal	100	100	100

Table 4
Regenerability of Thiolsulfonate Agarose (TS-Gel) and Thiolsulfinate-Agarose (TSI-Gel)

Gel type	Gel-bound SH groups μmoles SH/g dried gel	(%)	μMoles thiol reactive groups/g dried gel
SH-gel (starting point)	712	100	0
TSI-gel	<10	–	388
Regenerated SH-gel (after TSI gel)[a]	683	96	0
TS-gel	<10	–	382
Regenerated SH-gel (after TS-gel)[a]	370	52	0

[a]The regeneration was carried out after glutathione immobilization

Table 5
Reuse of TSI-Gel

Step	Gel bound SH groups μmoles SH/g dried gel	Thiol-reactive groups μmoles/g dried gel	Bound BSA (mg/g dried gel)
SH-gel	1027	–	–
TSI-gel (first use)	0	415	133
TSI-BSA derivative, after DTT	1083	0	5.5
TSI-gel (third use)[a]	0	545	132
TSI-BSA derivative after DTT	–	0	19

[a]After each use, elution was carried out and then the gel was regenerated.

33. In order to demonstrate the feasibility of reusing the support, several cycles of immobilization, elution of bound protein, regeneration of thiol-reactive adsorbent (TSI-gel), and reuse is performed. As high amounts of a model thiol-protein are required, bovine serum albumine-SH, provided with 4.0 moles of *de novo* thiol groups per mole of protein through chemical modification with SPDP, is selected for this purpose (**Table 5**).

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18

Very Strong But Reversible Immobilization of Enzymes on Supports Coated With Ionic Polymers

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Summary

In this chapter, the properties of tailor-made anionic exchanger resins based on films of large polyethylenimine polymers (e.g., molecular weight 25,000) as supports for strong but reversible immobilization of proteins is shown. The polymer is completely coated, via covalent immobilization, the surface of different porous supports. Proteins can interact with this polymeric bed, involving a large percentage of the protein surface in the adsorption. Different enzymes have been very strongly adsorbed on these supports, retaining enzyme activities. On the other hand, adsorption is very strong and the derivatives may be used under a wide range of pH and ionic strengths. These supports may be useful even to stabilize multimeric enzymes, by involving several enzyme subunits in the immobilization.

Key Words: Polymeric beds; volume effect; nondistorting but strong adsorption; reversible immobilization.

1. Introduction

Many protocols for enzyme immobilization involve the irreversible covalent binding between the enzyme and a pre-existing support. Usually, when the immobilized enzyme becomes inactivated during its industrial application both the enzyme and the support should be eliminated as wastes ***(1–8)***. In this way, these conventional protocols for enzyme immobilization have two important drawbacks: (1) a relatively high cost resulting from the use of large amounts of expensive supports and the performance of more or less complex immobilization protocols; and (2) the production of large amounts of waste when the immobilized derivatives become inactivated (such as the inactivated enzymes covalently immobilized on polymeric resins). Hence, these protocols for enzyme immobili-

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zation are only economically sustainable when the immobilized derivative is very stable and can be reused for many reaction cycles for the catalysis of high value-added processes.

From this point of view, the reversible immobilization of enzymes on pre-existing supports could be a very convenient protocol for the immobilization of many industrial enzymes. Thus, reversibility means the possibility of promoting the complete desorption of the enzyme away from the support when the immobilized derivative becomes inactivated and it cannot be further used in the industrial reactor. The supports can then be recovered fully intact and protein free, and they become ready to be used again for a new immobilization of a fresh solution of soluble enzyme. The support, even a fairly expensive one, can be used indefinitely, and the only waste produced is a solution of inactivated enzyme. We can summarize a number of advantages of such methods for reversible immobilization of industrial enzymes (e.g., via physical adsorption of enzyme on activated supports ***(1–12)***:

1. Activated supports are chemically inert and hence they are very stable during transport, storage.
2. Protocols for immobilization are usually very simple and carried out under very mild conditions.
3. After enzyme inactivation, the enzyme can be desorbed away from the support and this support can be reused for many times with the subsequent reduction of costs and wastes.
4. Complex enzyme reactors such as monolith reactors for treatment of large effluent volumes can be "*in situ*" regenerated after enzyme inactivation.

A number of protocols for this reversible immobilization of industrial enzymes have been reported in recent years ***(9–15)***. However, the most popular, simplest, and oldest protocol for reversible immobilization of enzymes is the adsorption of enzymes on ionic exchange resins. In fact, more than 25 yr ago, the first industrial process catalyzed by immobilization of enzymes (resolution of D,L-amino acids by amino acylase) was developed using this type of immobilized preparation ***(6,7)***. This reversible immobilization of enzymes is frequently fairly mild, and most of the proteins are therefore fully desorbed from such matrices at moderate and even low ionic strength (0.2–0.3 *M* NaCl). In this way, high concentrations of ionizable substrates, changes of pH during the reaction, and so forth can promote undesirable leakage of immobilized enzyme away from the support, promoting an apparent inactivation of the enzyme derivative and a certain contamination of the product ***(1–4)***.

The criteria for the selection and development of new anion exchange supports for immobilization of industrial enzymes are very different from the most adequate criteria to design supports for purification of enzymes and proteins by ion exchange chromatography. Approaches, immobilization, or purification may have very different requirements:

1. The key objective of enzyme and protein chromatography is the recovery of fully intact enzymes and proteins after adsorption on the support. The supports should be designed to permit a mild desorption of native proteins.

2. The key objective of the reversible immobilization of industrial enzymes is the prevention of enzyme leakage during catalytic operation and the further recovery of fully intact supports after enzyme inactivation. Therefore, the promotion of a very strong adsorption (and simultaneously, a difficult desorption) of the proteins on the supports is now very convenient. Desorption of the enzymes has only to be performed after enzyme inactivation and hence the mild recovery of intact proteins is not necessary. Now, the necessity of using drastic conditions such as very high ionic strength or extreme pHs for the desorption of inactivated proteins preserving supports is not a problem but it becomes an important advantage.

   With all of this in mind, it has been proposed that the preparation and selection of a new generation of matrices for a very strong ionic adsorption of enzymes is very suitable for the design of reversible immobilization of industrial enzymes ***(9–12)***. Composites based on the full coating of rigid porous supports with flexible polymers containing a very high concentration of ion exchange moieties are proposed as new matrices that are very promising to get a mild and very strong adsorption of industrial enzymes (although very inadequate for protein purification; *see* **Fig. 1**). In this way, the adsorption of industrial enzymes on a very high concentration of ionic exchange moieties placed on a flexible bed that is coating a rigid surface may be much stronger than adsorptions promoted on conventional ionic exchange resins (rigid surfaces containing a much lower concentration of ionic exchange moieties).

Supports activated with different groups (e.g., glyoxyl, amino, epoxy) can be covalently coated with different ionic polymers such as polyethyleneimine, polyalylamine, and aldehyde–aspartic dextran. Supports with ionic groups (e.g., carboxymethyl, sulfonic, amino) may be ionically coated with polymers of the opposite charge (e.g., amino support with sulfate–dextran). The coating conditions (e.g., time, polymer size and concentration, pH, and ionic strength) have been proven to be critical to this goal, because a polymeric bed formed by the full coating of the surface with very flexible immobilized ionic polymers is desired (*see* **Figs. 2** and **3**) ***(9–12)***.

2. Materials

1. Sepabeads® EC-EP (epoxy-activated supports) and Sepabeads-MANAE (primary amino-activated supports) supplied by Mitsubishi Chemical Co. (Milano, Italy).
2. Sulfate–dextran (different molecular weights) supplied from Sigma Chemical Co. (St. Louis, MO).
3. Polyethylenimine (PEI; different molecular weights) from Sigma.
4. Carboxymethyl cellulose (CMC) from Sigma.
5. Sulfate-dextran coating buffer: 25 m*M* sodium phosphate buffer, pH 7.0.
6. Sodium periodate supplied by Merck (Darmstadt, Germany)
7. Sodium borohydride supplied by Sigma.
8. Immobilization buffer: 5 m*M* sodium phosphate buffer 5 m*M*, pH 7.0, or 5 m*M* sodium acetate buffer, pH 5.0.
9. Desorption buffers: 5 m*M* sodium phosphate buffer at different pH values (from 5.0 to 9.0) with different NaCl concentrations.

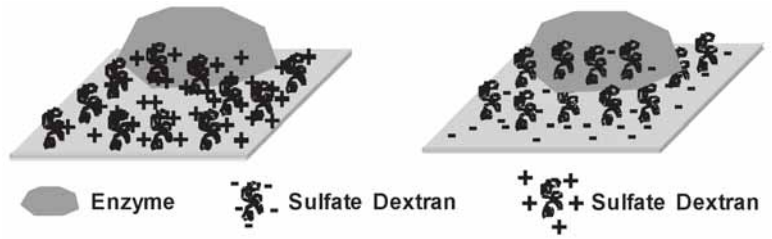

Fig. 1 . Strong and no distorting reversible immobilization of enzymes on sulfate–dextran supports or on polyethyleneimine supports.

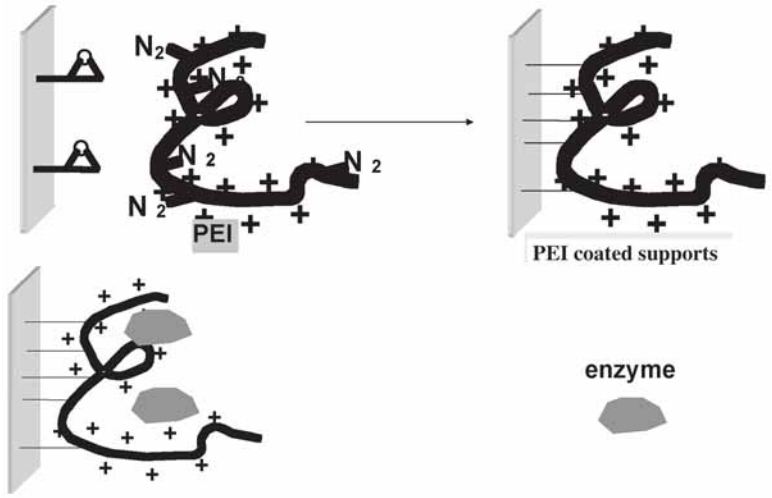

Fig. 2. Coating process of epoxy-Sepabeads with polyethyleneimine.

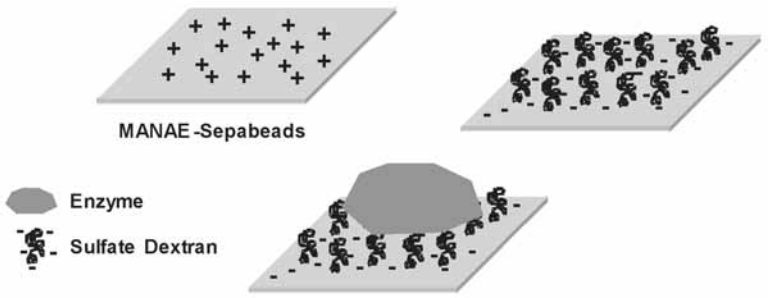

Fig. 3. Coating process of MANAE-Sepabeads with sulfate–dextran.

10. Washing solution to prepare PEI support: 100 m*M* sodium acetate buffer, pH 4.0, and 100 mM sodium borate buffer, pH 9.0, and water.
11. Washing solution to prepare sulfate–dextran support: 100 m*M* sodium phosphate buffer, pH 7.0, and water.

3. Methods

3.1. Preparation of Polyethyleneimine Support

1. Epoxy supports were incubated in 10 volumes of 100 m*M* sulfuric acid for 24 h to open all epoxy groups. Then, the support was equilibrated with 0.1 *M* sodium phosphate, pH 7.0, in batch during 2 h, and then filtered and washed with an excess of distilled water. Until the washing keep the initial pH value.
2. The support was added to 10 volumes of 50 m*M* sodium periodate for 2 h. Then, the aldehyde-support was washed with an excess of distilled water.
3. Next, aldehyde-supports were incubated at pH 11.0 with 10 volumes of 10% PEI (w/v) for 12 h under mild stirring at 25°C.
4. The support was reduced by adding 20 mg of solid sodium borohydride/mL of suspension during 1 h.
5. After, the PEI-supports were washed with 100 m*M* sodium acetate buffer, pH 4.0, and 100 m*M* sodium borate buffer, pH 9.0. Finally, the PEI–Sepabeads complex was washed exhaustively with distilled water.

3.2. Preparation of Sulfate–Dextran Supports

1. 10 g MANAE-Sepabeads were incubated in 20 mL of dextran–sulfate solution (8 g of dextran–sulfate [500 kDa] dissolved in 20 mL of 25 m*M* sodium phosphate buffer, pH 7.0) for 16 h at 25°C, under mild stirring.
2. Then, the composites were washed with 1 L of 100 m*M* sodium phosphate buffer, pH 7.0, under mild stirring for 1 h to decrease sulfate–dextran viscosity.
3. After that, the composites were washed with abundant water to eliminate the excess of sulfate–dextran.

3.3. Immobilization/Adsorption of Proteins on Ionic Exchanger Supports

1. For each of the different supports 5 g were suspended in 25 mL of an enzymatic solution (1 mg/mL or 1 U/mL was the maximum concentration in order to avoid diffusion problems) in sodium phosphate 5 m*M* at pH 5.0 or 7.0 at 20°C. The suspension was stirred gently.
2. Periodically, samples of supernatant and suspension were taken and the enzymatic activity and/or the protein concentration was measured by using the Bradford method.
3. After the immobilization process the preparations were washed with the same buffer use in the immobilization process.

3.4. Desorption of Proteins on Ionic Exchanger Supports

1. 5 g of composites containing the immobilized proteins were suspended in 5 m*M* sodium phosphate buffer, pH 5.0 to 9.0. Increasing concentrations of NaCl were then added.

2. Samples were taken from the supernatant 30 min after the NaCl addition (longer incubations times up to 3 h did not result in significant increments in the desorbed protein) and the protein concentrations were determined by Bradford method. When studying enzymes, desorption was checked via enzyme activity determinations. A reference solution with soluble enzyme was submitted to the same treatment to detect any possible effect the NaCl upon activity of the enzyme. The percentage of desorbed enzyme is referred to the amount of adsorbed one.

3.5. Thermal Stability Studies

1. To study the thermal stability of the enzyme derivatives, 1 U/mL was used. The inactivations were carried out at pH 5.0 or 7.0 and at different temperatures ranging from 37 to 70ºC.
2. Samples were withdrawn at different times and their activities were tested as previously described. The remaining activity was calculated as the ratio between activity at a given time and the activity at zero time of incubation.

3.6. Stability Studies in the Presence of Cosolvents

1. To study the stability of enzyme preparations in the presence of dioxane an enzyme concentration of 1 U/mL was used. Inactivations were carried out at pH 7.0 in the presence of different percentages of cosolvents at 25°C.
2. Samples were withdrawn at different times and their activities were tested as previously described. The remaining activity was calculated as the ratio between activity at a given time and activity at zero time of incubation.

3.7. Immobilization of Some Industrial Enzymes on Polymeric Supports

3.7.1. Immobilization of β-Galactosidase From Aspergillus oryzae on Polyethyleneimine Support

1. β–Galactosidase from *A. oryzae* preparation (*see* **Subheading 3.3.**)**.** was incubated on PEI-Sepabeads (*see* **Subheading 3.1.**).

   Figure 4 shows the immobilization course of β-galactosidase from *A. oryzae* onto the optimized PEI support. The immobilization proceeded very rapidly (almost all enzymes were immobilized in less than 1 h), whereas the activity of the immobilized enzyme was kept completely unaltered after the immobilization.

   Figure 5 shows that β-galactosidase from *A. oryzae* remained fully adsorbed on the PEI support even when it was incubated in 150 m*M* NaCl, whereas most enzymes (around 90%) were released to the medium using commercial DEAE-support at only 90 m*M* NaCl. In fact, 20% of the β-galactosidase from *A. oryzae* remaining adsorbed on PEI support even at 1 *M* NaCl. This immobilized preparation retained more than 80% of the immobilized activity after incubation for one month in 25 m*M* sodium phosphate, pH 7.0, at 37°C. Therefore, this derivative seemed to have quite good properties if it was to be used under very different conditions. The desorption process was carried out as described in **Subheading 3.4.**

3.7.2. Immobilization of Chymotrypsin From Bovine Pancreas on Sulfate–Dextran Support

Chymotrypsin from pancreas was offered (*see* **Subheading 3.3.**) onto the sulfate–dextran (*see* **Subheading 3.2.**) and CMC supports at pH 7.0. The chymot-

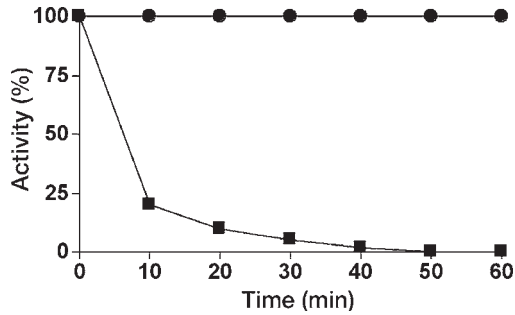

Fig. 4. Immobilization of β-galactosidase from *A. oryzae* on optimized PEI support. Immobilization experiment was carried out at pH 7.0 at 4°C in 5 m*M* sodium phosphate buffer: (●) activity of the suspension; (■) activity of the supernatant. The experiment was carried out as described in **Subheading 3.**

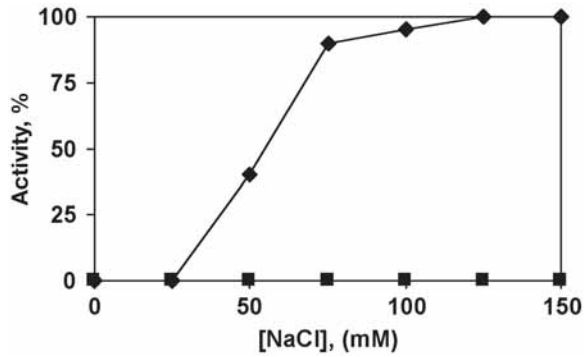

Fig. 5. Desorption of β-galactosidase from *A. oryzae* adsorbed on different anion exchanger supports at growing ionic strenghth. (■) Percentage of β-galactosidase activity released from optimized PEI support. (◆) Percentage of activity released from DEAE–agarose. The experiments were carried out as described in **Subheading 3.**

rypsin preparation (*see* **Subheading 3.3.**) was incubated with sulfate–dextran support in immobilization buffer (5 m*M* phosphate buffer, pH 7.0). The enzyme was rapidly immobilized onto both supports, keeping its activity completely unaltered (*see* **Fig. 6**).

Only 35% of adsorbed chymotrypsin on sulfate–dextran was released when it was incubated with 300 m*M* NaCl at pH 7.0, whereas the chymotrypsin adsorbed on CMC was fully released under these conditions (*see* **Fig. 7**).

In all cases, these facts seemed to suggest that this immobilization system was very mild for the enzyme structure, most likely because of the possibility of the polymer becoming adapted to the enzyme instead of forcing the enzyme to be adapted to the support ***(9–12,17)***.

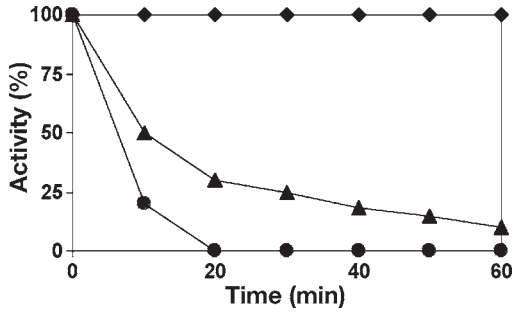

Fig. 6. Immobilization course of chymotrypsin from bovine pancreas on optimized Sulfate–dextran support and carboxymethyl support. Immobilization experiment was carried out at pH 7.0 at 4°C in 5 m*M* sodium phosphate buffer. The percentage of adsorbed proteins were measured at pH 7.0 at 25°C as described in **Subheading 3.** (◆) Activity of all suspensions; (▲) activity of the supernatants during immobilization of chymotrypsin on CMC; (●) activity of supernatants during the immobilization of chymotrypsin on optimized sulfate–dextran support.

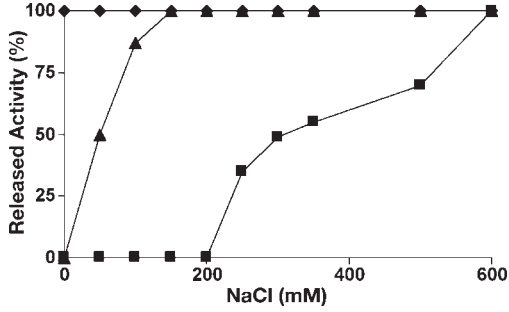

Fig. 7. Desorption of chymotrypsin from bovine pancreas adsorbed on different cation exchanger at growing ionic strength. Immobilization was performed at pH 7.0 in 5 m*M* sodium phosphate buffer as described in **Subheading 3.** The enzyme was released from supports by incubation on growing concentration of NaCl. (◆) Activity of all suspensions; (▲) activity released from CMC; (■) activity released from optimized sulfate–dextran composites. The experiments were carried out as described in **Subheading 3.**

3.8. Stability of Immobilized Enzymes on Polymeric Supports

3.8.1. Stability of Immobilized Multimeric Enzymes Immobilized on Ionic Polymer Coated Supports

Invertase from *Saccharomyces cerevisiae* was immobilized in 5 m*M* sodium phosphate, pH 7.0, on DEAE–agarose or PEI supports (*see* **Subheading 3.3.**), with an immobilization yield higher than 90% in both cases, after only 5 min of enzyme–support contact. The activity was fully preserved during the immobilization

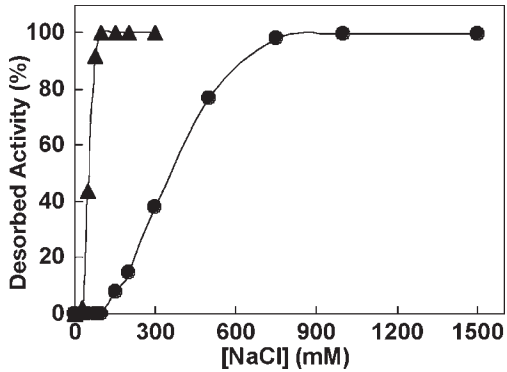

Fig. 8. Desorption of invertase from *S. cerevisae* adsorbed on different anion exchanging supports at growing ionic strength. (▲) Activity released from DEAE-agarose; (●) activity released from Sepabeads-PEI by incubation with increasing NaCl concentrations. Enzyme was immobilized at pH 7.0 as described in **Subheading 3.**, with a protein loading of 25 mg/g of support. Desorption was performed at pH 7.0.

protocol in both cases. The salt concentration at which the enzyme began to be desorbed from the support was much higher using PEI supports than a DEAE support (*see* **Fig. 8**). Although all the enzyme was desorbed from DEAE–agarose at only 100 m*M* of NaCl, invertase began to be partially desorbed from the PEI support at more than 200 m*M* of NaCl (*see* **Subheading 3.4 .**).

The invertase from *S. cerevisiae* remained fully active at pH levels between 5.5 and 8.5 at 4°C for 24 h. The immobilization could therefore be performed onto PEI supports in this wide range of pH values.

The immobilization rate was very high in all cases and the maximum load of the support was similar (around 30–35 mg of enzyme per gram of support), with activity retentions higher than 95% in all cases.

The stability of the soluble or immobilized (*see* **Fig. 9A**) enzyme at acidic pH values was higher than the stability at alkaline pH values. Curiously, the stability of the enzyme immobilized at pH 8.5 was higher than that of the enzyme immobilized at pH 5.5 when both preparations were inactivated at pH 4.5 at either 50°C, 55°C (*see* **Fig. 9B**), or 60°C (*see* **Subheading 3.5.**).

This result suggests that the different immobilized enzymes could present some differences promoted by the pH value of the immobilization. It has been described that invertase presents different compositions of multimeric structures when the pH value is changed ***(18–20)***. Also, it has been reported that PEI-coated supports may permit the stabilization of multimeric enzymes by implicating several subunits in the immobilization ***(9)***. Thus, if the enzyme presented a different aggregation form during the immobilization, this structure seems to be preserved after the immobilization.

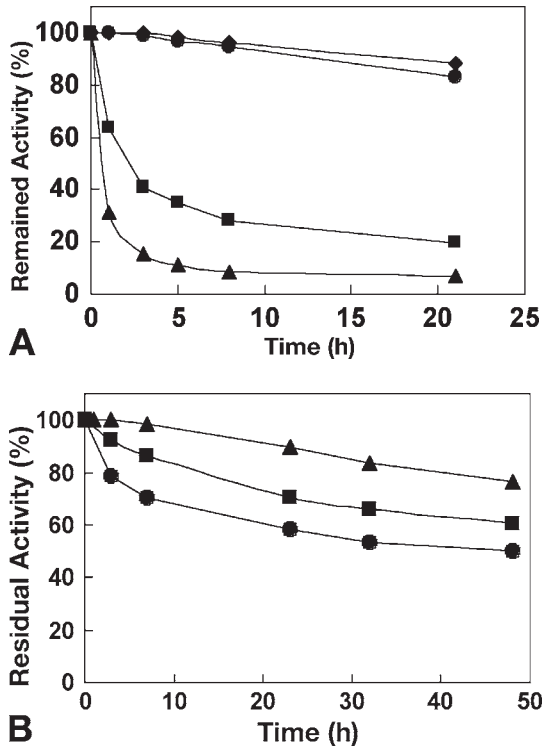

Fig. 9. **(A)** Effect of pH on the stability of invertase Sepabeads EC-EP3-PEI immobilized preparations. (◆) pH 5.0; (●) pH 5.5; (■) pH 7.0; (▲) pH 8.5. Enzyme was immobilized at pH 7.0 as described in **Subheading 3.**, with a protein loading of 25 mg protein/g of support. Inactivation of enzyme preparations was carried out at 55°C. **(B)** Thermal stability of invertase Sepabeads EC-EP3-PEI preparations immobilized at different pH values: enzyme load was 25 mg of protein/g of support. (▲) Immobilized enzyme at pH 8.5; (■) enzyme immobilized at pH 7.0; (●) enzyme immobilized at pH 5.5. Inactivation of immobilized enzyme was performed at pH 4.5 and 55°C.

3.9. Stability in the Presence of Cosolvents of Immobilized Enzymes on Polymeric Supports

In the presence of organic media, all industrial enzymes immobilized on sulfate–dextran supports exhibited certain stabilization. These results show that the use of ionic polymers such as sulfate–dextran may generate a hydrophilic microenviroment around the enzymes, preventing their inactivation by partition of the organic solvent molecules. **Figure 10** shows that trypsin and chymotrypsin immobilized on the sulfate–dextran support (*see* **Subheading 3.3.**) were also much more stable against the action of organic solvents compared to the CMC immobilized preparation. The stability studies were carried out as described in **Subheading 3.6.**

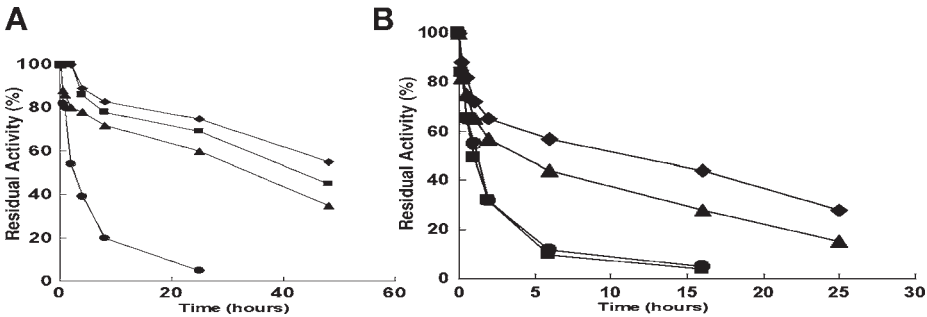

Fig. 10. Inactivation of different chymotrypsin and trypsin from bovine pancreas sulfate–dextran derivatives in the presence of cosolvents. Immobilization was performed at pH 7.0 on optimized sulfate–dextran support as described in **Subheading 3.** Inactivation of derivatives was performed at pH 7.0 at 25°C in the presence of different percentage of dioxane. Other specifications are described in **Subheading 3. (A).** Stability of trypsin derivatives in presence 30% dioxane. (◆) Optimized sulfate–dextran support immobilized derivative at pH 7.0. (■) Optimized sulfate–dextran support immobilized at pH 5.0. (▲) Carboxymethyl support immobilized derivative at pH 7.0. (●) Carboxymethyl support immobilized derivative at pH 5.0. (**B)** Stability of chymotrypsin derivatives in presence 50% dioxane. (◆) Optimized sulfate-dextran support immobilized derivative pH 7.0. (▲) Optimized sulfate–dextran support immobilized derivative pH 5.0. (■) Carboxymethyl support immobilized derivative at pH 7.0. (●) Carboxymethyl support immobilized derivative at pH 5.0.

4. Notes

1. PEI viscosity is high, so the 10% PEI solution (w/v) requires a gentle stirring during preparation.
2. Viscosity of sulfate–dextran solution is high. It is therefore necessary to wash exhaustively with abundant water to eliminate excess.
3. To immobilize on PEI supports it is necessary to first wash the PEI support with immobilization buffer in order to balance the pH on the support and prevent the buffer effect caused by the ionic polymer, which could change the pH, and avoid the enzyme immobilization or promote enzyme inactivation.
4. To immobilize on sulfate–dextran supports it is necessary to first wash the sulfate–dextran support with immobilization buffer in order to balance the pH on the support and prevent the buffer effect caused by the ionic polymer, which could change the pH, and avoid the enzyme immobilization or promote enzyme inactivation.
5. The polymer size is an important parameter because a large size is necessary to wrap up the proteins. Moreover, the polymer size is strongly related with protein size.

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19

Immobilization of Enzymes on Magnetic Particles

Martina Koneracká, Peter Kopčanský, Milan Timko, Chenyl Nynitapal Ramchand, Zainul M. Saiyed, Michael Trevan, and Anil de Sequeira

Summary

Magnetic particles have been increasingly used as carriers for binding proteins, enzymes, and drugs. Such immobilization procedures for proteins, enzymes, antibodies, and other biologically active compounds have a major impact in different areas of biomedicine and biotechnology. The immobilized biomolecules can be used directly for a bioassay or as affinity ligands to capture or modify target molecules or cells. This chapter details immobilization procedures for proteins and enzymes onto various magnetically responsive carriers such as naked magnetic particles, carboxyl-modified microspheres, and amino-modified microspheres using direct binding procedure in the presence of coupling agents such as carbodiimide. The physical and chemical properties of freshly prepared magnetic particles were determined by magnetic measurements (VSM magnetometer), transmission electron microscopy (TEM), and atomic force microscopy (AFM). The extent of immobilization and enzyme activities were spectrophotometrically measured in order to find the retained activity after immobilization onto magnetic particles. The binding of proteins and enzymes was also confirmed by TEM microscopy.

Key Words: Albumin; glucose oxidase; chymotrypsin; streptokinase; dispase; carbodiimides.

1. Introduction

Immobilization of proteins, enzymes, antibodies, and other biologically active compounds onto inert solid support has found several applications in the field of biotechnology and biomedicine. Conventionally, enzymes and cells were immobilized on a solid support, such as synthetic polymeric matrices, or entrapped within the gel matrix formed of different polysaccharides such as alginate, cellulose, or agarose. The use of synthetic polymers as supports for enzyme immobilization provides several advantages (e.g., inertness to microbial attack, higher chemical resistance, and the option to use complex buffer components). Of the many synthetic polymers, polystyrene (PS) is inexpensive, readily available in different par-

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ticle sizes, and possesses mechanical rigidity ***(1)***. Nonporous PS-based adsorbents have been employed for reverse-phase ***(2)***, anion-exchange ***(3)***, and affinity chromatography of proteins ***(4–6)***. Whereas the use of gel matrices for enzyme immobilization offers a relatively inert aqueous environment within the matrix, the high gel porosity allows for high diffusion rates of macromolecules. Alginate beads have been used for a variety of biotechnological applications ***(7)***.

In recent years, several magnetic separation procedures have been developed to isolate and purify biomolecules ***(8)***. The advantage offered by such techniques is the ease of recovery, speed, and extreme specificity by which a biomolecule can be isolated from a complex mixture. Over the past decade, systems containing micrometer- or submicrometer-sized magnetic particles coated with a suitable stabilizer have been used as carriers for binding proteins, enzymes, and drugs. For example, heparin-stabilized colloidal magnetite has been used for binding of cells from whole blood; dextran-coated magnetite has been used as a drug carrier, whereas silane-coated ferrite particles are used for radioimmunoassays ***(9)***. Magnetic particle-based systems have also been used as an affinity matrix for separation of enzymes. One-step purification of horse liver alcohol dehydrogenase by magnetic Sepharose® beads coupled with 5'-AMP has been successfully accomplished ***(10)***. Magnetic alginate microsphere have been used for affinity purification of α-amylase ***(11)***.

Superparamagnetic microspheres preconjugated to various types of generic binding proteins (such as Protein A and streptavidin) and antibodies are rapidly becoming the solid-phase support of choice in many areas, including immunological applications, nucleic acid work, and cell separation and visualization. These monodisperse spheres are composed of magnetite in a polymer matrix. The polymer beads are then coated with functional polymers that provide reactive groups, such as COOH and NH_2. Proteins can be adsorbed readily onto PS microspheres or covalently coupled to carboxylic acid or other functional groups on the surface of microspheres. Covalent coupling is, however, preferable for many applications. A covalently bound ligand is likely to be more stable, to cover the surface more completely or more evenly, and to be more efficient in terms of consumed reagents. Furthermore, addition of spacers or linkers will allow biomolecules to be presented in a more flexible fashion, and careful chemistry can attach ligands in a specific orientation ***(12)***. Antibodies can be modified in a similar manner. Antibody-coated microspheres form the basis for particle capture enzyme-linked immunosorbent assay (ELISA) tests and assays. Antibodies immobilized onto magnetic beads have been utilized extensively in diagnostics and other research applications for the purification of cells and biomolecules. Magnetite labeled antibodies is expected to be applicable clinically as therapeutic agents for the induction of hyperthermia ***(13)***.

Most manufacturers provide variously sized particles that have a variety of different chemical terminations. For example, dextran-based biocompatible magnetic nanoparticles are available commercially from Miltenyi Biotec, Germany. These magnetic beads (μMACS) are available with many covalently immobilized molecules such as protein A, protein G, streptavidin, and antibodies against various CD markers ***(14)***. Likewise, Polysciences, Inc., manufactures and supplies Biomag® magnetic beads consisting of an iron-oxide core with an inert silane coating. The particle surface is functionalized with amine or carboxyl groups for the

covalent attachment of proteins, glycoproteins, and other ligands with retention of biological activity. Biomag particles are supplied covalently attached to a wide variety of monoclonal antibodies (MAbs) specific for human and murine cell surface markers or secondary antibodies such as goat antimouse, goat antirat, and sheep antifluorescein ***(15)***.

Biologically active compounds immobilized on magnetic carriers can also be used directly for a bioassay or as affinity ligands to capture or modify target molecules or cells. For example, Abudiab and Beitle ***(16)*** have developed a magnetic immobilized metal affinity separation media for isolating proteins from complex mixtures. The method involved coupling of iminodiacetic acid (IDA) to the surface of magnetic agarose, this when charged with metal ions (Cu^{+2} or Zn^{+2}) is capable of binding model proteins that display metal affinity and of separating protein mixture ***(16).*** Similarly, Qiagen Inc., Germany has developed protein purification and assay protocols based on Ni-NTA magnetic agarose beads to suit different applications. Ni-NTA (nitriloacetic acid) tagged magnetic agarose beads have been used for versatile magnetocapture assays using 6× His-tagged proteins ***(17)***. The method employs both metal affinity and magnetism as the basis of purification. The procedure involves use of metal chelating NTA groups covalently bound to the surface of agarose beads, which contain strong magnetic particles. Nickel ions immobilized on NTA have a high affinity for a tag of six consecutive histidine residue, thereby allowing capture of 6× His-tagged proteins for sensitive interaction assays or microscale purification. Thus, using this technique purification of protein based on Ni-NTA metal chelate affinity followed by direct use of purified protein for microplate-based assays is possible ***(18)***. Other procedures for magnetic affinity separations of various proteins have been summarized recently ***(19)***.

Mehta et al. ***(20)*** have shown that proteins and enzymes can be bound covalently to freshly prepared magnetite in the presence of carbodiimide. Several clinically important enzymes and proteins that include bovine serum albumin ***(20)***, streptokinase, chymotrypsin, dispase, and glucose oxidase (GO) have been immobilized based on this method. The immobilized enzymes showed between 50 and 80% of the original added enzyme activity. The direct coupling method for enzymes or bioactive molecules to the magnetic particles is a result of the presence of hydroxyl group on the magnetic support. Bacri et al. ***(21)*** have shown that hydroxyl group will remain on the particles at pH between 6.0 and 10.0. Thus, the free hydroxyl group on the surface of the particles is responsible for the binding of the enzymes.

The direct coupling method for enzymes or bioactive molecules to the magnetic particles has a number of potential advantages. Because these magnetite particles are not coated with any polymer materials, the overall size is smaller, thus increasing the ratio of surface area to volume, allowing a greater response to any magnetic field. Moreover in case of cell separation using magnetic particles it is shown that the larger the particle size used for separation, the higher the extent of nonspecific entrapment in the larger aggregates of magnetic particles. Thus smaller magnetic particles hold the promise of greater specificity; because they can exist as stable colloidal suspensions that will not aggregate, thus allowing for uniform distribution in a reaction mixture ***(22)***. This chapter details immobilization procedure for proteins and enzymes onto magnetically responsive carriers.

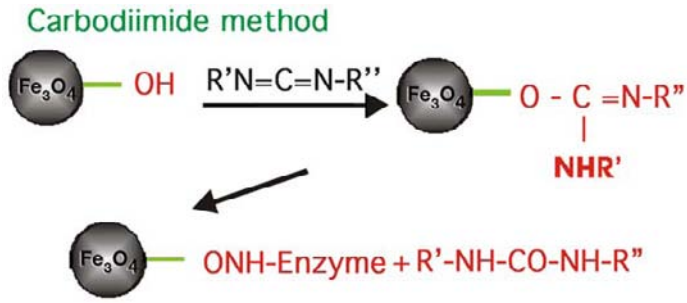

Fig. 1. Scheme of immobilization of enzyme protein to magnetic support via carbodiimide coupling agent.

1.1. Immobilization of Enzyme Protein to Magnetic Particles Using Direct Binding Procedure

A water-soluble carbodiimide derivative can be used to activate hydroxyl group on the surface of magnetic particles and thereby couple the amino group on the enzymes to the hydroxyl group through an amide linkage (*see* **Fig. 1**). It is important that the pH of the reaction medium is kept neutral. At a pH that is too low, the amine is protonated and does not react readily, whereas at a higher pH the carbodiimide may decompose. The coupling procedure is simple to perform and has the advantage of occurring under very mild conditions and of conjugating the enzymes directly to magnetic particles without interposing additional groups between two ***(20,22)***.

1.2. Physical and Chemical Properties of Magnetic Particles

The magnetic properties of magnetic particles are described as superparamagnetic (having zero coercivity and remanence) and the classical theory of Langevin paramagnetism, modified to include a particle size distribution, can be used. So the magnetization as a function of magnetic field in liquid phase can be expressed by following equation:

$$I(H) = I_s \int_0^\infty L(ax^3) f(x)\, dx \tag{1}$$

where $L(y) = coth(y) - 1/y$ is the Langevin function, $a = \pi I_s D_r^3 \mu_o H/6k_B T$, I_s is the saturation magnetization of the bulk material, $x = D/D_r$ is the reduced diameter (D_r is the mean diameter of particles) and $f(x)$ is the particle size distribution function. For the system of fine magnetic particles the particle size distribution function is log normal ***(23)***:

$$f(y) = \frac{1}{y\sigma\sqrt{2\pi}} \cdot e^{\frac{(\ln(y))^2}{2\sigma^2}} \tag{2}$$

where $f(y)$ is log-normal distribution function and σ is standard deviation.

The particle size distribution is possible to estimate from magnetization measurements and consecutively then relations for D_v and σ can be expressed as:

$$D_v = \left(\frac{18kT}{\pi I_s} \cdot \sqrt{\frac{\chi_i}{3I_s H_0}} \right)^{1/3} \quad (3)$$

$$\sigma = \frac{1}{3} \cdot \sqrt{1n \frac{3\chi_1}{I_s (1/H_0)}} \quad (4)$$

where χ_i is initial susceptibility.

For example, the saturation magnetization I_s was measured to be 14 mT, as using the above-mentioned method the log-normal parameters of the particle size distribution have been calculated to be: mean particle diameter D_v, of 9.3 nm, and standard deviation σ of 0.29. Using the relation $m = I_s V_r$ (V_r is mean volume of particle calculated from mean particle diameter and I_s is saturation magnetization of particle) the magnetic moment of the individual particle can be estimated. In our case for D_v = 9.3 nm the magnetic moment was estimated as to be 1.84×10^4 μ_B (μ_B is elementary Bohr magneton, which has the value $\mu_B = 9.2740789 \times 10^{-24}$ Am^{-2}). **Figures 2** and **3** provide the results from atomic force microscopy (AFM) and transmission electron microscopy (TEM) measurements, respectively.

From the results obtained by the various measurement techniques, it is clear that for the mean particle diameter values: $D_{v/VSM}$ (9.3 nm) < $D_{v/TEM}$ (12.2 nm) < $D_{v/AFM}$ (13.1 nm) and for the standard deviation values: σ_{VSM} (0.29) > σ_{TEM} (0.22) > σ_{AFM} (0.21). However, the smaller mean particle diameter observed from VSM data (9.3 nm) in comparison with value obtained from TEM and AFM data can be explained by the presence of a nonmagnetic surface layer around the magnetite particles and small differences in standard deviation can be caused by method of sample preparation ***(24)***.

The estimation of particle size and its distribution is very useful, as the physical and chemical properties of magnetic fluids depend on the detail of particle size distribution mainly at biological application of magnetic fluids.

2. Materials

2.1. Preparation of Magnetic Particles

1. Ammonium hydroxide, iron (II) sulfate heptahydrate, and iron (III) chloride hexahydrate were procured from Sigma-Aldrich (St. Louis, MO).
2. Vibrating sample magnetometer (VSM) was used for estimation of volume concentration of magnetite particles, saturation magnetizations, and cosequently for particle size diamater.

2.2. Immobilization of Proteins Onto Naked Magnetic Particles

1. Bovine serum albumin (BSA), GO, chymotrypsin, streptokinase, dispase, and 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride (CDI) were obtained from Sigma-Aldrich.
2. Coupling buffer: 3 m*M* sodium phosphate buffer, pH 6.3.

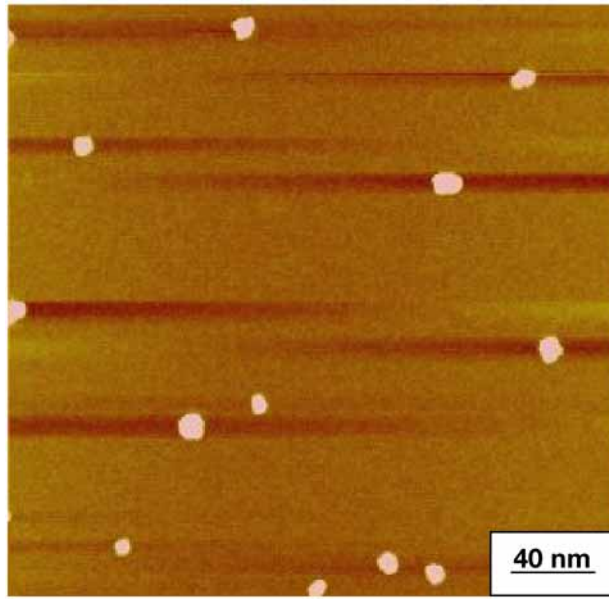

Fig. 2. AFM image of magnetite nanoparticles. The obtained values for mean diameter and standard deviation were D_v = 13.1 nm σ = 0.21.

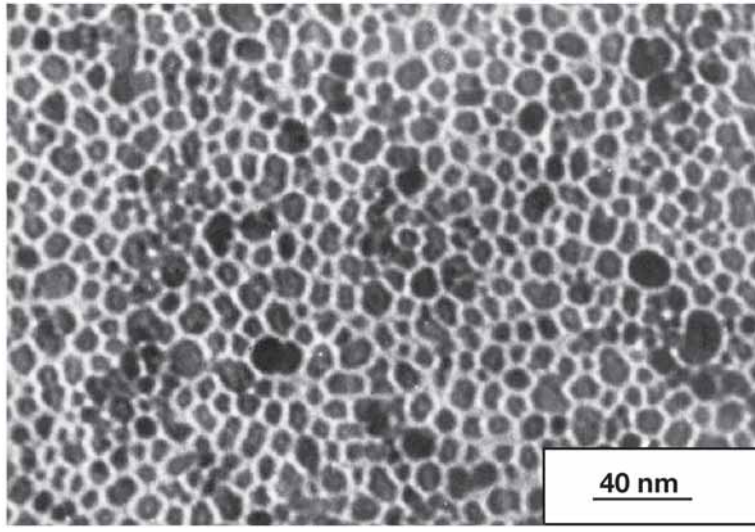

Fig. 3. TEM micrograph of magnetite particles. The obtained values for mean diameter and standard deviation were D_v = 12.2 nm and σ = 0.22, respectively.

2.3. Immobilization of Proteins Onto Carboxyl-Modified Microspheres (25)

1. Carboxyl-modified microspheres (often supplied at 10% solids; *see* **Note 2**).
2. Activation buffer, pH 4.5 to 7.5.
3. Coupling buffer, pH 7.2 to 8.5: 25–100 m*M* phosphate-buffered saline (PBS) (Buffers containing free amines, such as Tris of glycine should be avoided.)
4. Water soluble carbodiimide (WSC; e.g., 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride and/or 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate).
5. Protein or other biomolecule that is to be immobilized.
6. Quenching solution with primary amine source (30–40 m*M*; e.g., hydroxylamine, ethanolamine, glycine, or other) and 0.05–1% (w/v) blocking reagent (BSA, casein, pepticase, polyethylene glycol [PEG]).
7. Storage buffer, pH 7.0 to 7.5: 25–100 m*M* PBS, containing 0.01 to 0.1% blocking reagent (BSA, casein, pepticase, polyethylene glycol).

2.4. Immobilization of Proteins Onto Amino-Modified Microspheres (25)

1. Amino modified microspheres (often supplied at 10% solids; *see* **Note 2**).
2. Amine reactive homobifunctional cross-linker (e.g., glutaraldehyde, imidoesters, or NHS esters).
3. Wash/coupling buffer, pH 6.0 to 9.0: 25–100 m*M* PBS.
4. Protein and other biomolecules that is to be immobilized.
5. Quenching solution with primary amine source, 30–40 m*M* (e.g., hydroxylamine, ethanolamine, or glycine) with 0.05–1% (w/v) blocking reagent.
6. Storage buffer, pH 7.0 to 7.5: 25–100 m*M* PBS, pH 7.4, containing 0.01 to 0.1% blocking reagent (BSA, casein, pepticase, polyethylene glycol).

3. Methods

3.1. Preparation of Magnetic Particles

Magnetic particles (Fe_3O_4) were prepared by co-precipitating ferric and ferrous salts in an alkaline solution followed by washing in hot water (*see* **Fig. 4** and **Notes 3** and **4**).

1. Dissolve 27.8 g of iron (II) sulfate heptahydrate ($FeSO_4$ C $7H_2O$) and 54 g of Iron (III) chloride hexahydrate ($FeCl_3$ C $6H_2O$) in 100 mL double distilled water.
2. Mix the above solution thoroughly.
3. Add drop-wise to 8 *M* NH_4OH with constant stirring at room temperature.
4. Heat the obtained precipitate at 80°C for 30 min.
5. Black particles were obtained that exhibited strong magnetic response.
6. Wash the particles with copious amounts of hot distilled water.
7. Finally, magnetic particles were suspended in slightly alkaline medium, pH 8.9.

3.2. Immobilization of Different Proteins Onto Naked Magnetic Particles Using Carbodiimide (see *Fig. 5*)

Bovine serum albumin (BSA), GO, chymotrypsin, streptokinase, and dispase were immobilized onto magnetic particles using 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride (CDI).

1. Dissolve the protein that is to be coupled in phosphate buffer pH 6.3 at a concentration of 20 mg/mL.

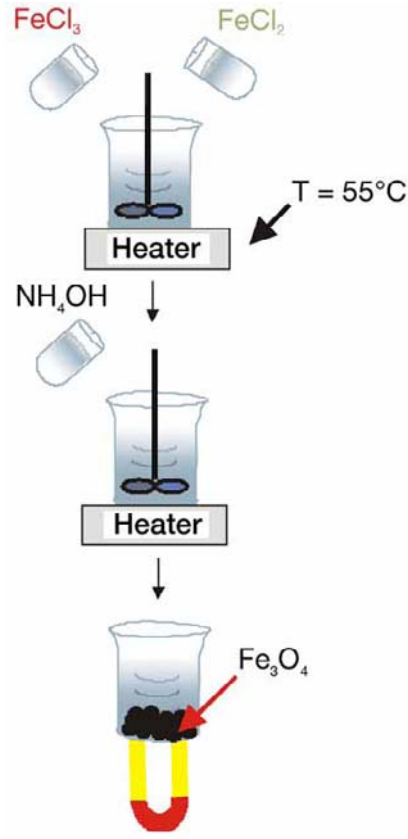

Fig. 4. Scheme for preparation of fine magnetic particles.

2. Separately weigh and dissolve CDI in phosphate buffer, pH 6.3, at a concentration of 20 mg/mL.
3. Mix 1 mL of protein (20 mg/mL) with 1 mL of CDI (20 mg/mL) dissolved in buffer, pH 6.3.
4. Add 3 mL of a given volume of magnetite particles that had previously been washed copiously in buffer, pH 6.3, to the mixture of protein and CDI.
5. Incubate the reaction mixture on a shaker at room temperature for 24 h.
6. After 24 h separate magnetic particles by placing a magnet under the vial containing the reaction mixture.
7. Decant the supernatant, wash magnetic particles three times in buffer solution.
8. Pool the supernatants, measure the left over protein concentration using Bradford's dye binding assay (*see* **Note 1**).
9. Calculate the quantity of bound protein as the difference between the total protein added to the immobilization mixture and the total protein recovered from the pooled washings.

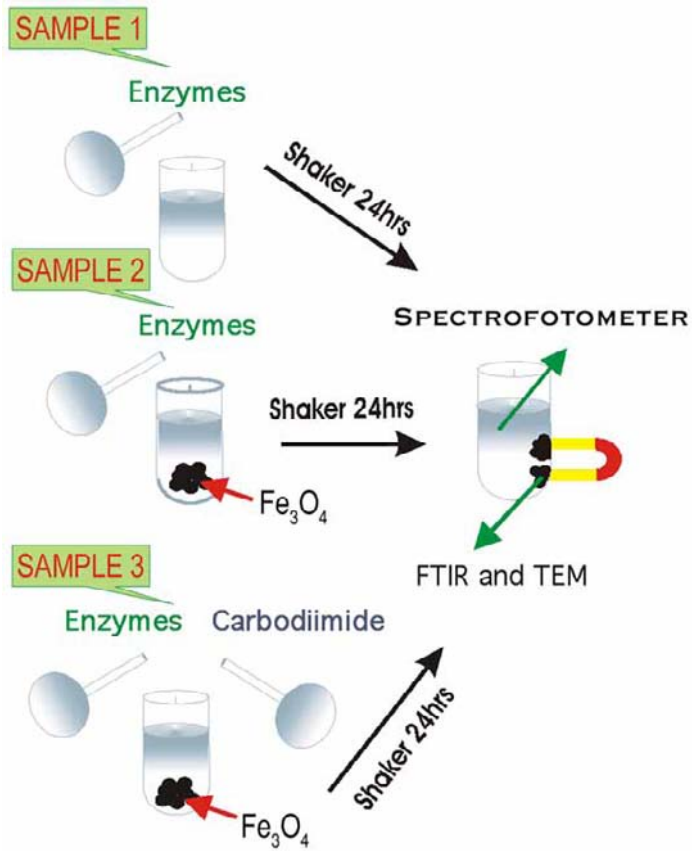

Fig. 5. Scheme for immobilization of enzymes onto fine magnetic particles.

10. Confirm the binding of protein to magnetic particles by FTIR spectroscopy and electron microscopy (*see* **Fig. 6**).
11. Check the enzyme activity in order to find the retained activity after immobilization onto magnetic particles (*see* **Notes 5–7**).

3.3 Immobilization of Proteins Onto Carboxyl-Modified Polymeric Microspheres (25)

1. Wash 1 mL (100 mg/mL) of microspheres two times in 10 mL of activation buffer (MES buffer is a common choice).
2. After washing, completely resuspend microspheres in 10 mL of activation buffer.
3. Add 100 mg of water soluble carbodiimide (WSC) to the microspheres with constant stirring.
4. Incubate the reaction mixture at room temperature for 15 min.
5. Wash microspheres two times with coupling buffer and resuspend completely in 5 mL of the same.

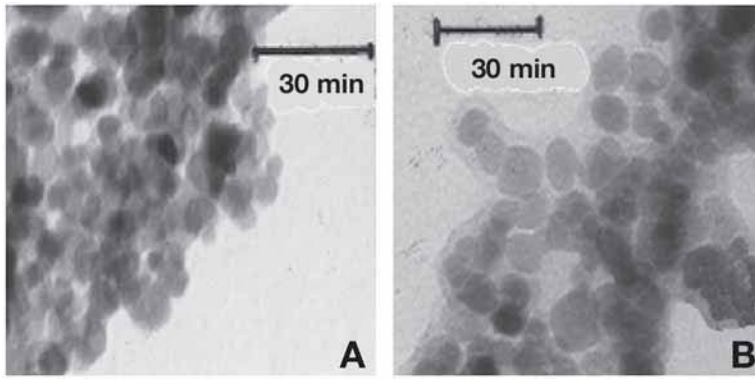

Fig. 6. The evidence of successful immobilization of BSA can be seen as thin layers around the magnetite particle **(B)**.

6. Dissolve protein (1–10X excess of calculated monolayer) in 5 mL of coupling buffer.
7. Combine microsphere suspension and protein solution.
8. Incubate at room temperature for 2 to 4 h with constant stirring.
9. Wash, resuspend in 10 mL of quenching solution, and mix gently for 30 min.
10. Wash and resuspend in storage buffer to desired storage concentration (often 10 mg/mL).
11. Store at 4°C until used.

3.4. Immobilization of Proteins Onto Amino-Modified Polymeric Microspheres (25)

1. Wash 1.0 mL (100 mg/mL) of microspheres twice with 10 mL of coupling buffer.
2. After washing, completely resuspend microspheres in 10 mL of glutaraldehyde solution (glutaraldehyde dissolved in wash/coupling buffer to a final concentration of 10%).
3. Incubate at room temperature for 1 to 2 h, with constant stirring
4. Wash twice and resuspend in 5 mL of wash/coupling buffer.
5. Dissolve protein (1–10X excess of calculated monolayer) in 5 mL of coupling buffer.
6. Combine microsphere suspension and protein solution.
7. Incubate the reaction mixture with constant stirring for 2 to 4 h at room temperature.
8. Wash and resuspend in 10 mL of quenching solution and mix gently for 30 min.
9. Wash and finally resuspend in storage buffer to desired storage concentration (often 10 mg/mL).
10. Store at 4°C until use.

4. Notes

1. Because of interference of carbodiimide Lowry's method for protein estimation should not be used.

2. Polymeric microspheres, produced commercially by Bangs Laboratories, are available with many surface active functional groups such as carboxyl and amino, as well as hydroxyl, hydrazide and chloromethyl; and silica–silanol. Biomolecules can be effectively linked to these microspheres through a variety of coupling chemistries. As examples, covalent coupling of proteins or other biomolecules using carboxyl and amino-modified microspheres has been described above (TechNote 205 Covalent Coupling; at http://www.bangslabs.com). Recently, Bangs Laboratories also introduced magnetic microspheres with different surface groups that are intended for covalent coupling of ligands such as protein, and nucleic acids.
3. The saturation magnetizations of dehydrated particles were determined with a vibrating sample magnetometer (VSM).
4. The amount of magnetic particles in a given volume of magnetic fluid was estimated thermogravimetrically and by magnetic measurements of magnetization curves (VSM magnetometer).
5. GO activity determined by GO–PO method.
6. Streptokinase activity determined by the smallest amount of streptokinase that causes lyses of a standard fibrin clot within 10 min.
7. Chymotrypsin and dispase assayed according to the Sigma diagnostic procedures ***(26)***.

Acknowledgment

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Immobilization of Proteins on Gold Surfaces

José M. Abad, Marcos Pita, and Víctor M. Fernández

Summary

Gold has been a widely used support for protein immobilization in a nonspecific way through electrostatic and hydrophobic interactions. As no tools are available to predict the binding of proteins of biological interest to gold supports—for either nano, micro, or macroscopic sizes—smart, reliable, and reproducible protein immobilization protocols on gold are sought. This chapter will focus on a synthetic strategy which allows for the development of a multiplicity of architectures on gold that may be used for protein immobilization. Because of its simplicity, both from a conceptual and a practical point of view, the strategy demonstrated by this step-by-step synthesis of a functionally self-assembled monolayer (SAM) of thiols on gold is accessible to most laboratories working on enzyme technology, even those with limited organic synthesis facilities.

Key Words: Gold; self-assembled monolayers; SAM; oriented immobilization; nanoparticles.

1. Introduction

Gold has been widely used as a support for proteins in different applications. A classic, well-known example is the use of colloidal gold particles as a support for antibodies in immunological studies to detect cell antigens at the optical and electron microscope levels. The most popular variant makes use of gold nanoparticles covered with protein A that specifically interacts with the *Fc* fragment of immunoglobulin (IgG) molecules; the use of protein A labeled with ^{125}I made this process a rapid quantitative method for estimation of membrane antigens ***(1)***. In this arrangement, protein A plays a dual role, as a linker of IgG to gold nanoparticles and as a stabilizer of colloidal gold solutions. The stability of gold nanoparticles in solution is maintained by electrostatic repulsions, and the addition of electrolytes can alter the surface charge of the nanoparticles and produce their flocculation. In addition, positively charged groups of the proteins interact with the negatively charged surface forming noncovalent binding complex and stabilizing the colloid from the effects of electrolytes ***(2)***.

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Proteins tend also to adsorb to macroscopic gold surfaces in a nonspecific way by electrostatic and hydrophobic interactions ***(3)***. Interestingly, proteins of a certain size that adsorb into a gold material may also bind to a smaller particle of the same material, but less strongly. Moreover, a polypeptide that binds preferentially to a specific crystallographic surface may bind with an altered affinity to another crystallographic surface of the same material ***(4)***. Progress made in the understanding of protein adsorption on gold was reported in the recent work by K.S. Shulten and colleagues ***(5)***. These authors have simulated the binding of a peptide engineered to match the spacing of the gold atomic lattice. Their calculations indicate that polar side chains of serine and threonine residues can establish contact with gold atoms of Au (111) surfaces, placing a periodic structure of OH^- groups on the regular lattice. The same peptides do not bind to Au (112) as tightly, because of the migration of water molecules through this crystallographic surface. However, despite these advances, no tools are available to predict the binding of proteins of biological interest to gold supports. Therefore smart, reliable and reproducible protein immobilization protocols on gold are sought.

Over the past years, there has been a noticeable interest in covering gold surfaces with monolayers of proteins based on the molecular recognition properties of biological systems ***(6)***. This strategy for the immobilization of proteins on surfaces while retaining their full activity and stability constitutes a challenge. Most of the common methods are difficult to control and usually yield randomly bound proteins. On the contrary, an ideal immobilization would produce saturation coverage of specifically bound proteins. The formation of protein layers is induced by anchoring the protein molecules to gold surfaces functionalized with active molecules, such as transition metal complexes for the immobilization of proteins with repetitive histidine sequences. A feasible method to uniformly cover gold surfaces consists of the self-assembly of thiols by oxidative-chemisorption over the gold ***(7)***. Reversible monolayers of histidine-tagged proteins have been produced using a gold layer covered with a monolayer of a chelator thioalkane ***(8)***. Other systems have been described with biotynilated alkanethiols ***(9)*** and this biotin-avidin system has been used as a template to direct the binding of monobiotynilated Fab fragments of monoclonal antibodies ***(10)***. As an alternative to this methodology, which demands highly complex organic synthetic work, a step-by-step construction of the functional monolayer over a template of thiocarboxylic acid chemisorbed onto gold has been worked up ***(11)***. In a comparative study of *N*-palmitoylcysteamine self-assembled monolayers (SAMs), either from *ex situ* synthesis of the chain or from *in situ* acylation of a cysteamine SAM ***(12)***, the authors did not find differences in topography and mean roughness between the two monolayers.

These studies have benefited from the development of surface sensitive techniques for the detection of bimolecular interactions between one partner immobilized on a gold surface and the complementary molecule from the solution. The binding effect can be monitored by specific signals associated to the binding event. Representative examples of detection systems on gold-modified surfaces are atomic force microscopy (AFM) ***(13)***, quartz crystals microbalance (QCM) ***(14)***, and surface plasmon resonance (SPR) ***(15)***.

Because of its simplicity, both from a conceptual as well as a practical point of view, the step-by-step synthesis of a functional self-assembled monolayer is accessible to most of the laboratories working on enzyme technology, even those with limited facilities for organic synthesis. This chapter focuses on the synthetic strategy, which by a judicious design of the synthetic route allows the development of a multiplicity of architectures on SAMs (**Fig. 1**).

We have used this strategy for controlled immobilization of enzymes onto gold surfaces and used them as amperometric electrodes in the characterization of their catalytic performance ***(14,16,17)***.

2. Materials

2.1. Preparation of Piranha Solution

1. Sulfuric acid, 95 to 98%, American Chemical Society (ACS)-grade reagent (Aldrich). **Caution:** Corrosive and toxic.
2. Hydrogen peroxide, 30% solution in water. **Caution:** Corrosive and toxic.
3. Fume cupboard, disposable gloves, and face protection.

2.2. Gold Support

1. 0.5-mm Gold wire, 99.99% (Goodfellow, Cambridge, UK).
2. Gamma Micropolish Alumina B 0.05 micrometer (Buehler).

2.3. Gold Modification With Thiol Self-Assembled Monolayers Presenting Reactive Ester Intermediates

1. Thioctic acid (6,8-dithiooctanoic acid, TOA) reagent (Sigma).
2. Ethyl alcohol–Water (2:1 [v/v]) solution.
3. Dimethyl sulfoxide (DMSO; Merck). **Caution:** Higly flammable; harmful.
4. *N*-Hydroxysuccinimide (NHS; Sigma).
5. 1-Ethyl-3-[3-(dimethylaminopropyl)] carbodiimide (Sigma).

2.4. Coupling of Spacers

1. 1,8-Diamino-3,6-dioxaoctane (DADOO; Merck).
2. Dimethyl formamide (DMF).
3. Ethylenediamine (Sigma).
4. 1-Chloro-2,3-epoxypropane (epichlorohydrin; Epi) (Fluka). **Caution:** Toxic by inhalation or contact with the skin; may cause cancer; flammable.

2.5. Coupling of Affinity Ligands

1. Thiocholine (ThC; MP Biochemicals, Irvine, CA).
2. *N*-(5-Amino-1-carboxypentyl)-iminodiacetic acid (ANTA) (Fluka).
3. 3-Aminophenyl boronic acid (Aldrich).

2.6. Enzymatic Activity

1. (*p*-Aminophenyl)-β-D-galactopyranoside (PAPG) (Sigma).
2. NADPH (Sigma).
3. β-Aminoethyl ferrocene, synthesized as described by Godillot et al. ***(18)***.
4. 4-Aminophenol hydrochloride (Sigma).
5. Thionine (Lauthe Violett, Merck).

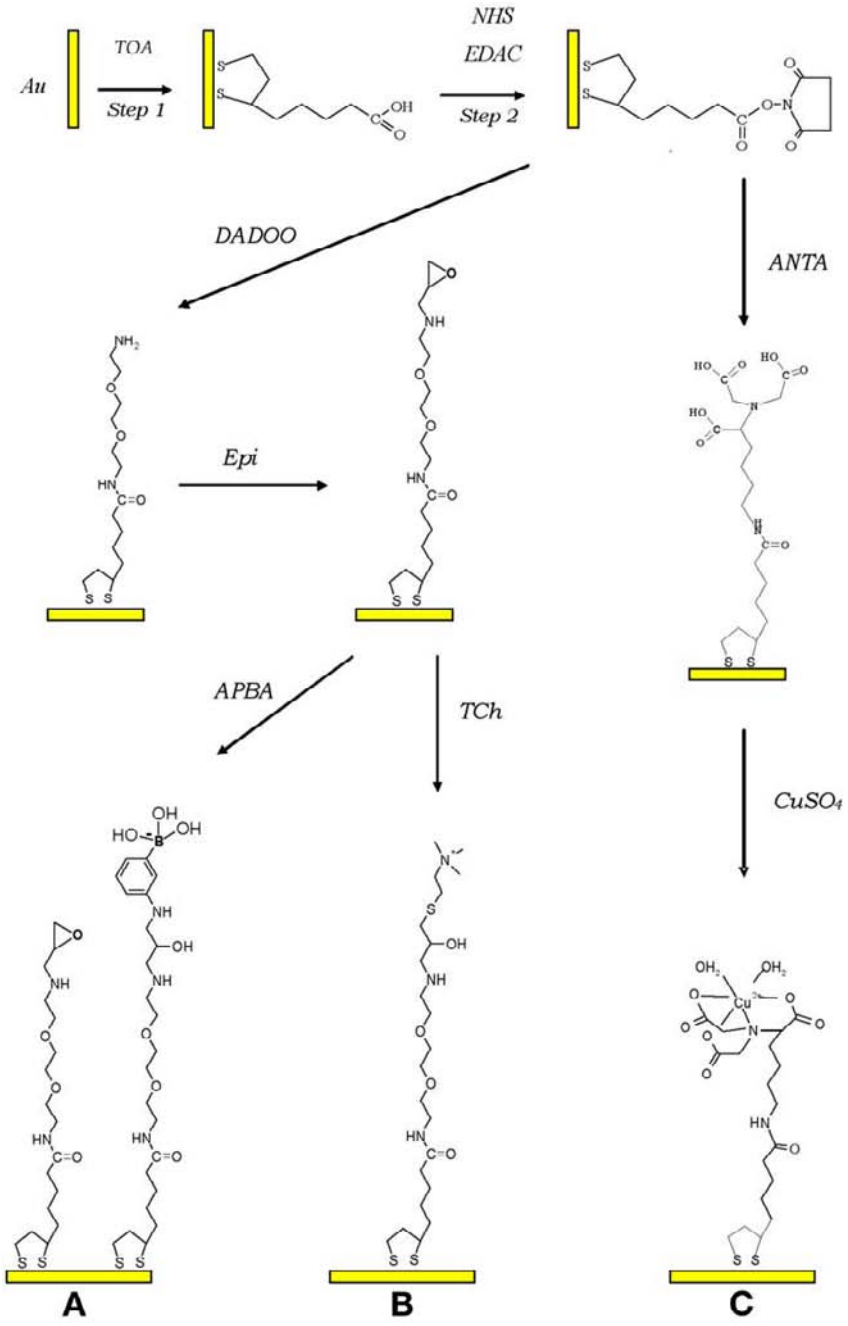

Fig. 1. Step-by-step synthesis of functional SAMs onto gold surfaces for specific protein immobilization. (**A**) SAM: TOA–DADOO–[Epi:APBA]. (**B**) SAM: TOA–DADOO–EpiThC. (**C**) SAM: TOA–ANTA–Cu(II).

2.7. Enzymes

1. The fusion protein of β-gal and C-Lyta was produced and purified as reported previously ***(19)***.
2. Ferredoxin $NADP^+$ reductase native and mutants purified as described ***(16)***.
3. Type 1 horseradish peroxidase (Sigma).

3. Methods

3.1. Preparation of Piranha Solution

1. Using gloves and protective glasses, operate in the fume cupboard.
2. Mix 98% sulfuric acid with 30% hydrogen peroxide solution, in a 3:1 volume ratio. The acid is slowly poured onto the peroxide.
3. This solution is explosive and corrosive. It reacts violently with organic matter, so it should be handled with extreme caution. Be very scrupulous with the safety indications.
4. Keep this solution inside the fume cupboard far from flames.

3.2. Gold Support Preparation

1. Heat a 0.5-mm gold wire directly on a Bunsen burner until the gold reaches light red temperature, but avoid melting.
2. Polish the heated wire with γ-alumina suspension of 0.05-μm diameter for about 10 min.
3. Wash the wire with Milli-Q water.
4. Dip the wire in a 2:1 [vol/vol] ethanol–water solution.
5. Sonicate the wire in an ultrasonic bath for 15 min.
6. Air-dry.

3.3. Gold-Surface Modification With Thioctic Acid Monolayers

1. Dip the gold wire in a 1 m*M* DL-6, 8-TOA solution prepared in ethanol–water solution (2:1 [v/v]) at room temperature for 24 h.
2. The gold electrode, covered with a dithioctic SAM is rinsed in ethanol–water for 5 min and then air-dried.

3.4. Activation of Carboxylic Acid Groups of the Monolayer

1. The TOA-modified gold is immersed for 3 h in a solution of 0.1 *M N*-hydroxysuccinimide (NHS) in DMSO, containing 0.1 *M* 1-ethyl-3-[3-(dimethyl-amino)] propyl carbodiimide (EDAC), which catalyzes the esterification reaction.
2. Rinse the gold electrode for 5 min with DMSO while stirring gently with a magnetic stirrer.
3. Air-dry the electrode.

Up to this step, the process is general for all the SAMs that will be described further. From now on, the chemical modification is different for each type of SAM that will be synthesized. In the following subheadings the step-by-step synthesis of different monolayers decorated with ligands chosen by their affinity for specific protein motifs is described.

3.5. Reversible Immobilization of a Chimeric Protein Obtained by Genetic Fusion of a Choline-Binding Domain With β-Galactosidase on a Choline Self-Assembled Monolayer

3.5.1. Coupling of a Hydrophilic Spacer

For proper attachment of the protein the setting of a spacer molecule between the SAM and the protein is needed to facilitate the accessibility of biotin to the binding pocket on the protein.

1. The amidation of NHS-esters previously synthesized on the SAM is effected by the overnight immersion of the electrode into undiluted DADOO.
2. The unreacted diamine is removed by washing with ethanol followed by dipping in 0.1 *M* KOH.
3. As an alternative, the synthesis of a mixed SAM of DADOO and ethanolamine (EA) can be useful to prepare electrodes in which the DADOO spacers are diluted with shorter hydroxy-ending molecules. In this case the gold electrode is not immersed in DADOO but in DADOO–EA mixtures of both liquids. The DADOO-EA molar-ratio can be chosen as desired, but 1:5 is preferred. For this mixed monolayer, the electrode is washed in the same way as that for the DADOO-only one.

3.5.2. Activation of Terminal Amino Groups of TOA–DADOO Gold Electrodes

1. Activate the end free-amine group of DADOO by dipping the gold electrode in undiluted epichlorohydrin for 3 h.
2. Eliminate the unreacted epichlorohydrin by washing with ethanol–water solution (2:1 [v/v]) for 5 min.

3.5.3. Thiocholine Coupling to the Electrode Activated With a Self-Assembled Monolayer TOA-DADOO-Epi

1. The terminal epoxy groups generated in the previous step are dipped for 18 h with a 10 m*M* solution of ThC in 50 m*M* phosphate buffer, pH 8.0.
2. Wash the wire for 5 min with the same buffer.

3.5.4. Binding of a Chimera Protein Made of Fused β-Galactosidase and Choline-Receptor

1. To minimize the nonspecific adsorption of the chimeric protein dip the modified gold electrode for 10 min in a 0.5 mg/mL lactoalbumin solution.
2. Wash the wire with 50 m*M* sodium phosphate buffer, pH 7.3, for 10 min.
3. Incubate the washed wire in a 0.1 mg/mL chimeric protein solution in the same buffer.
4. Wash the electrode with the same buffer, as in **step 2**, for 2 to 3 min.

3.5.5. Measurement of the Enzymatic Activity

The course of the enzymatic reaction was followed by cyclic voltammetry (CV) analysis of the reaction product 4-aminophenol ***(20)*** (*see* **Note 1**). In this experi-

mental set up the gold wire serves as both enzyme support and electrode reporter of catalytic activity.

1. Insert the enzyme-SAM-modified wire in a conventional electrochemical cell with 2 mL of 50 m*M* phosphate buffer, pH 7.3, and 0.1 *M* KCl. Insert a platinum auxiliary electrode and an Ag/AgCl, 3 *M* NaCl reference electrode, connected to the cell by a salt bridge filled with the electrolyte solution.
2. Connect the cell to a electrochemical analyzer such as the CV50, BAS analyzer.
3. Add the substrate (p-aminophenyl)-D-galactopyranoside (PAPG) to a final concentration of 4 m*M* and immediately start to record cyclic voltammograms at 0.1 V/s in the potential interval from –0.2 V to +0.35 V, at intervals from 0 to 12 min.
   a. Plot the oxidation peak current at 0.14 V against time.
   b. Calibrate the electrochemical system by recording cyclic voltammograms at 0.1 V/s in the potential interval from –0.2V to +0.35V of 4-aminophenol hydrochloride solutions in a range from 0 to 5 mM. The current intensities of the aminophenol oxidation peak are plotted against concentration. Slopes about 45 µA/µmol/cm are obtained under the above experimental conditions.

3.5.6. Reversibility of the Chimera Protein Binding

1. Removal of the chimeric protein from the electrode is obtained by incubation of the enzymatic electrode in a 3 *M* choline solution; after 10 min incubation, no activity is detected.
2. Wash the electrode with 50 m*M* phoshate buffer, pH 7.3, 0.1 *M* KCl, and the electrode is ready for immobilization of a new chimeric protein sample.

3.6. Reversible and Oriented Immobilization of His-Tagged Proteins by Metal Complex Affinity

3.6.1. Amidation of the Thioctic Acid With Nitriolacetic Acid

Starting from the SAM configuration described in **Subheading 3.4.**, SAMs of dithioctic acid activated with the NHS, the electrode is modified with *N*-(5-amino-1-carboxypentyl)iminodiacetic (ANTA), a compound that forms stable octahedral complex with Cu(II), Ni(II), Co (II) and Zn(II), while leaving two vacant sites for coordination of nucleophiles like histidines.

1. Incubate overnight the gold covered with a SAM–TOA–NHS in a 0.15 *M* solution of ANTA in DMF/H_2O (2:1). Alternatively, a mixture of ANTA and EA can be used in a molar ratio range from 1 to 0.1 (total concentration of amine reactive group, 0.15 *M*) to prepare electrodes where the ANTA ligands were diluted with shorter hydroxy-ending molecules.
2. Wash the electrodes with DMF.

3.6.2. Metal Chelating of Nitriloacetic Acid

For a copper chelate, this step consists on dipping the modified electrode in a 40 m*M* $CuSO_4$ solution, pH 5.5, (acetate buffer 50 m*M*) for 1 h. If cobalt is desired, work the same way with 40 m*M* $CoCl_2$ salt instead of the copper one. All solutions must be prepared with Millipore (Milli-Q plus) water (18.2 MΩ · cm).

3.6.3. Immobilization of His-Tagged Ferredoxin-NADP Reductase

1. Incubate the gold wire with its surface covered by SAM–TOA–NTA–Cu^{2+} in 50 m*M* phosphate buffer solution containing 5 m*M* imidazole and 1.4 μ*M* of FNR mutant with exposed histidine pairs.
2. Wash the electrodes with 50 m*M* phosphate buffer and 0.15 *M* KCl, pH 7.5.

3.6.4. Measurement of the Enzymatic Activity

The enzyme ferredoxin-$NADP^+$ reductase catalyzes the oxidation of NADPH with artificial electron acceptors (diaphorase activity ***[21]***). Therefore, the course of the enzymatic reaction was followed by CV analysis of the reaction product, using the same methodology described in **Subheading 3.5.4.** above.

1. Insert the FNR–SAM-modified gold wire in a conventional electrochemical cell with 2 mL of 50 m*M* phoshate buffer, pH 7.5, 0.1 *M* KCl, and 20 μ*M* ferrocenemethanol. Insert a platinum auxiliary electrode and a Ag/AgCl, 3 *M* NaCl calomel electrode, connected to the cell by a salt bridge filled with the electrolyte solution.
2. Connect the cell to a electrochemical analyzer such as the CV50, BAS analyzer.
3. Add the substrate NADPH to a final concentration of 2 m*M* and immediatedly start to record b bcyclic voltammograms at 10 mV/s . The current-potential curve shows a plateau.
4. Wash the cell and repeat **steps 1–4** in the absence of NADPH. Subtract the plateau current from the current measured in **step 4**. The net current is proportional to the activity of the enzyme in the electrode.
5. Repeat **steps 1–5** at different concentration of substrates to estimate the kinetic constants according to the methodology described by Bourdillon et al. ***(22)*** (*see* **Note 2**).

3.6.5. Reversibility of the Chimeric Protein Binding

Incubation of the protein monolayer in 100 m*M* imidazol solution to release the FNR from the electrode.

3.7. Covalent Immobilization of Glycosylated Proteins Previously Adsorbed Through Weak, Reversible Reactions on a Mixed Epoxi-Boronic Acid (APBA) Self-Assembled Monolayer

Starting from electrodes as in **Subheading 3.4.**, boronic acids are incorporated into SAMs to reversibly adsorb glycosylated proteins through their carbohydrate moiety. A mixed monolayer of boronates and epoxy groups makes the adsorption irreversible. Basically, the aminophenylboronic groups promote and accelerate the covalent attachment of glycoproteins through the epoxy groups.

3.7.1. Amidation of the Thioctic Acid With Aminophenyl Boronic Acid and Epoxy Groups

1. Incubate overnight the gold covered with a SAM–TOA–NHS in undiluted DADOO.
2. Remove the unreacted DADOO by washing with ethanol.
3. Dip the gold electrode in undiluted Epi to produce a monolayer of epoxy groups.
4. Dip the electrode in ethanol 5 min to eliminate unreacted Epi. Repeat the wash with ethanol.

5. Incubate Epi-terminated electrodes in 0.16 *M* APBA in acetonitrile:water (1:5), pH 8.0, for 8 h. This protocol produces mixed monolayers with phenyl-boronate and epoxy groups.
6. To obtain an estimation of the remaining epoxy groups on this mixed SAM, incubate the electrode overnight in 2 m*M* β-aminoethyl ferrocene in DMF. Eliminate unreacted β-aminoethyl ferrocene by washing with solvent.
7. Insert the electrode in the electrochemical cell and run a cyclic voltammogram in the range of 150 to 500 mV. The integrated charge of the anodic cyclic voltammetry peak allows the calculation of remaining epoxy groups.

3.7.2. Immobilization of Horseradish Peroxidase

1. Incubate the modified electrode in 0.2 mg/mL HRP solution in 50 m*M* bicarbonate buffer for 24 h.
2. Wash the electrode for 30 min in the same buffer.

3.7.3. Measurement of the Enzymatic Activity

The enzyme peroxidase catalyzes the reduction of H_2O_2 to H_2O with an electron donor (e.g., thionine). In the assay, a 0.01 m*M* thionine in 0.05 *M* phosphate buffer, KCl 0.1 *M*, pH 7.5, is reduced electrochemically and supplies electrons to the peroxidase enzyme molecule, which in its catalytic cycle donates two electrons to H_2O_2. Therefore, the course of the enzymatic reaction can be followed by CV analysis of the reduced thionine reaction product, using the same methodology described in the reference.

4. Notes

1. This reference corresponds to a multiauthored and comprehensive treatise of electronalytical techniques.
2. We used protein engineering to control the immobilization of FNR molecules in two different orientations. Depending on the orientation of the immobilized protein on the monolayer the rate of electron transfer and NADH oxidation could be modulated.

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21

Immobilization of Enzymes on Electrodes

Gilvanda Silva Nunes and Jean-Louis Marty

Summary

This chapter focuses on the four main immobilization techniques used in the development of enzyme electrodes: adsorption, entrapment, covalent coupling, and cross-linking. The performance of the immobilization method depends on the enzyme and each method has both advantages and drawbacks. Loss of the activity results from the change in enzyme conformation depending on the method of immobilization as well as modification of the microenvironment (i.e., local pH of the enzyme). The activity of the immobilized enzyme decreases as a result of barrier of diffusion, which slows down or prevents the substrate from reaching the active site of the enzyme. The development and the characteristics of an acetylcholinesterase biosensor are described

Key Words: Enzyme immobilization; conformation; microenvironment; acetylcholinesterase; biosensor.

1. Introduction

Enzymes can be operated in the liquid form or immobilized on various supports. In enzymatic biosensor engineering, the term *immobilized* means "unable to move" or "stationary," but the enzyme activity remains still in the analytical device, although in a generally minor extension when compared with free enzymes ***(1)***.

The main advantages that accompany immobilized enzymes and many methods for immobilization are reduced cost of operation (as compared with free enzyme systems, which require additional separation and purification steps); the higher enzyme stability in immobilized form; the ability to stop the reaction rapidly by simply removing the enzyme from the reaction solution (or vice versa); the fact that products are not contaminated with the enzyme (and vice versa); the elimination of either multiple or repetitive use of a single batch of enzymes; and the possibility of establishing a model system to study enzyme action with variable applications. Among the disadvantages are the greater expense as compared to preparing free enzymes (depending on the immobilization method and on the

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reagents used), the lower activity exhibited by many immobilized enzymes when compared with free ones, and limitations in mass transfer because of the chosen immobilization method.

The use of biosensors based in immobilized enzymes for analytical purposes is ever increasing. The longer mean-life of the obtained enzymatic systems, the predictable decay rates, and the possibility of the elimination of sample preparation have made the enzymatic biosensors preferable and particularly useful in the food and pharmaceutical industries ***(2)***.

1.1. Enzyme Immobilization Methods

There are four basic methods that have been used in biosensors: (1) adsorption, (2) entrapment, (3) covalent coupling, and (4) cross-linking. The characteristics and advantages of these four methods are outlined in **Table 1**. The cross-linking method is the most frequently used method for variable applications because it has the advantage of covalent bonding in a simple and inexpensive procedure. These methods are explained and detailed in the following chapter.

1.2. Physical Methods

1.2.1. Adsorption

Physical adsorption of an enzyme onto a solid matrix is probably the simplest and fastest way to prepare immobilized enzymes. The method relies on a nonspecific physical interaction—based on weak forces, such as van der Waals or dispersion forces—between the enzyme and the surface of the matrix which is brought about by mixing a concentrated solution of enzyme with the solid ***(3)***. The active site is normally unaffected and a nearly full activity is observed.

Numerous enzymes have been immobilized though adsorption procedures. The main advantage of adsorption as a general method for insoluble enzymes is that usually no reagents, and only a minimum of activation steps, are required. As a result, adsorption is cheap, easily carried out, and tends to be less disruptive to the enzyme protein than chemical means of attachment. Because of the weak bonds involved, desorption of the protein (resulting from changes in temperature, pH, and ionic strength) appears to be the main problem. Another disadvantage is nonspecific adsorption of other proteins or substances ***(1,3)***.

1.2.2. Microencapsulation (Outer Membrane Entrapment)

This method is based on confinement of the enzyme in a membrane that is placed on an electrode surface. This membrane retains the enzyme and presents controlled porosity in order to allow free diffusion of substrate and reaction products through it (i.e., semipermeability of the membrane). Numerous membranes have been used, such as nylon, cellulose nitrate, cellulose acetate, epoxy resins, collagen, polysulfones, polyacrylates, and polycarbonates. Many disadvantages are associated with the mass transfer phenomena of substrates, reaction products, and inhibitors through the membrane, as well as with microorganisms growing on the membrane surface, leading to inhibition of the enzyme layer and causing the sensor to behave erroneously ***(3)***.

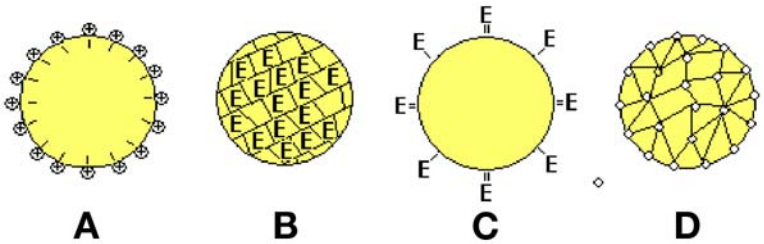

Table 1
Comparison of the Four Basic Enzyme Immobilization Methods

Characteristics	(A) Adsorption	(B) Entrapment	(C) Covalent coupling	(D) Crossl-inking
Matrix material	*Inorganic supports:* ion exchange resins, active charcoal, silica gel, clay, aluminum oxide, titanium, diatomaceous earth, hydroxyapatite, ceramic, celite, treated porous glass. *Organic supports:* starch, collagen, modified sepharose, CM cellulose.	Alginate, carageenan, collagen, polyacrylamide, gelatin, silicon rubber, polyurethane, polyvinyl alcohol with styrylpyridinium groups.	Agarose, cellulose, PVC, ion exchange resins, porous glass.	*Cross-linking reagents*: glutaraldehyde, *bis*-isocyanate, *bis*-diazobenzidine, diazonium salts. Functionally inert proteins, such as ovoalbumin and BSA, are often used as binder elements.
Bonding nature	Reversible; changes in pH, temperature, and ionic strength may detach the enzyme.	Physical entrapment	Chemical bonding (diazotation, peptic bond, alkylation, bonding with polyfunctional agents).	Entrapment
Enzyme loading	Low	Low	High	High
Enzyme leakage	Some	Some	Very low	Low
Loss of enzyme activity	Negligible	Negligible	Significant	Small
Cost	Inexpensive	Inexpensive	Expensive	Inexpensive
Operational	Simple and non-destructive technique; some instability (attributable to enzyme desorption).	Easy control and non-destructive technique, possible diffusion barriers; some enzyme losses.	High stability; absence of diffusion barriers; low response time; high enzymatic charge; low reproducibility.	Higher enzyme activity loss; operational facility; high enzymatic charge.

1.2.3. Occlusion Methods

Methods based on confinement of the enzyme within the lattices of a polymerized matrix or into intersticial spaces of a gel have also been used for enzyme immobilization. Such methods allow for free diffusion of low-molecular-weight substrates and reaction products. Numerous materials have been used for the enzyme occlusion, such as polyacrylamide gels, polyvinyl alcohols (PVAs), charged polymers, and cationic and anionic groups ***(3)***.

Generally, this immobilization method is useful for all types of enzymes (e.g., dehydrogenases, alcohol oxidase, cholinesterase, choline oxidase, glucose oxidase, tyrosinase). Moreover, it is simple, inexpensive, and results in relatively stable systems. The main disadvantages associated with this method are losses in enzyme activity by denaturation resulting from the presence of free radicals and/or by the ultraviolet radiation applied on the electrodes after enzyme immobilization in order to accomplish polymerization. These problems can affect the biosensor response and decrease the electrode stability.

In the last few years, immobilization methods based on sol-gel matrices have been used extensively for the construction of biosensors, mainly because gel materials present specific properties that are particularly interesting for biosensor construction (i.e., rigidity, thermal and photochemical stabilities, chemical inertness, and functionality in aqueous and organic media) ***(4)***. **Figure 1** exemplifies the enzyme occlusion by polymerization in PVA with styrylpyridinium groups (PVA-SbQ) and by using a sol-gel matrix.

1.2.4. Electro-Polymerization

Enzymes can be also physically immobilized into conductor polymers such as polypyrrole, polyaniline, polyphenols, and polythiophenes. Electrochemical potentiostatic and galvanostatic polymerization of the monomers in the presence of an enzyme produces a very sensitive and thin layer. The advantages associated to this technique are: an immobilization process that is entirely controlled, the use of several commercially available electrode materials (e.g., platinum, gold, carbon), and the possibility of miniaturization. Despite its operational simplicity, this procedure has one main disadvantage—it is difficult to determine the exact amount of the immobilized enzyme ***(5)***.

1.2.5. Electrode Modification

Enzyme immobilization can be performed also by simply adding the enzyme to a commercial activated graphite or epoxy—graphite containing some mediator. This is done in order to obtain a sensitive paste that will be incorporated into the working electrode surface. Obtained electrodes have excellent operational stability and high half-life ***(6)***.

1.3. Chemical Methods

1.3.1. Covalent Bonding

The most extensively studied of the insolubilization techniques is the formation of covalent bonds between the enzymes and the support matrix. This is the retention of the enzyme on support surfaces by covalent bonding between functional groups on the enzyme and those on the support surface. The active site of the

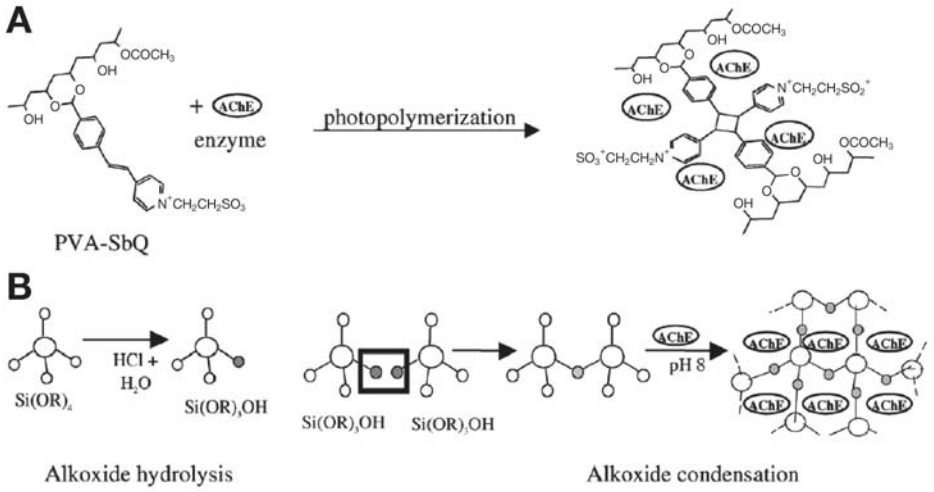

Fig. 1. Schematic representation of two occlusion methods for enzyme immobilization: polymerization in PVA with styrilpirydinium groups (PVA-SbQ) **(A)** and enzyme entrapment in a sol-gel matrix **(B)**.

enzyme must not participate in covalent bonding so that enzyme inhibitors may be added to the enzyme solution during covalent bonding treatment ***(3)***. When trying to select the type of reaction by which a given protein should be insolubilized, the choice is limited by the fact that the binding reaction must be performed under conditions that do not cause loss of enzymatic activity, and as previously mentioned, the active site of the enzyme must be unaffected by the reagents used.

The functional groups of proteins suitable for covalent binding under mild conditions include: the α-amino groups of the chain and the ε-amino groups of lysine and arginine; the α-carboxyl group of the chain; the β- and γ-carboxyl groups of aspartic and glutamic acids; the phenol ring of tyrosine; the thiol group of cysteine; the hydroxyl groups of serine and threonine; the imidazol group of histidine, and the indol group of tryptophane.

A small number of reactions have been designed to couple with functional groups on the protein other than the amino and phenolic residues. Amino ethyl cellulose has been coupled to the carboxylic acid residues of enzyme protein in the presence of carbodiimide, and thiol residues of a protein have been oxidatively coupled to the thiol groups of a cross-linking copolymer of acrylamide and *N*-acryloyl-cysteine. It is possible in some cases to increase the number of reactive residues of an enzyme in order to increase the yield of insolubilized enzyme and provide alternative reaction sites to those essential for enzyme activity. As with cross-linking, covalent bonding should provide stable and insolubilized enzyme derivatives that do not leach enzyme into the surrounding solution. The wide variety of binding reactions and insoluble carriers with functional groups capable of covalent coupling (or of being activated to give such groups) makes this a reasonably applicable method of insolubilization, even if very little is known about the protein structure or active site of the enzyme to be coupled.

There are many commercially available pre-activated membranes that have been used as supports for covalent enzyme fixation ***(7)***. Nevertheless, this enzyme immobilization method is generally the most complicated and difficult to reproduce. Moreover, the quantity of enzyme to be immobilized is usually higher.

1.3.2. Reticulation

Glutaraldehyde can also lead to the reticulation of enzyme molecules, creating an insoluble, reticulated, and rigid net. Reticulation can be performed in the presence of an inert protein (e.g., bovine serum albumin [BSA]) in order to increase the stabilization of the immobilized enzyme. This immobilization procedure is called reticulation and offers advantages such as solidity of enzyme–enzyme and enzyme–protein couplings. Unfortunately, reticulation can induce the formation of diffusion barriers, which can result in a higher biosensor response time.

1.3.3. Enzyme Fixation in Self-Assembled Monolayers

More recently, enzyme fixation of biomolecules on self-assembled monolayers (SAMs) has been used for biosensor construction. This method allows for controlled and oriented enzyme immobilization, thus being preferable for application in immobilization of enzymes on metallic electrodes such as gold, platinum, and silver ***(8)***.

In practice, certain molecules (e.g., thiolalkanes and silanes) are used for electrode surface activation, and monolayer formation takes place directly on it. Such monolayers utilize bifunctional molecules for the enzyme immobilization through covalent bonding or specific linking (e.g., enzyme affinity or recognition protein–ligand process). In this mode, acid functions can be activated by succinimide or carbodiimide groups and amine groups can be activated by glutaraldehyde ***(3,8)***.

1.4. Effects of Immobilization

In order to decide which immobilization technique to use it is first important to understand the changes in physical and chemical properties that an enzyme would be expected to undergo upon immobilization. Changes have been observed in the stability of enzymes and in their kinetic properties because of the microenvironment imposed upon them by a supporting matrix and also by the products of their own action. There is usually a decrease in specific activity of an enzyme upon immobilization that leads to the enzyme insolubilization; this can be attributed to denaturation of the enzyme protein caused by the coupling process.

With immobilized enzymes, the measured reaction rate depends not only on the substrate concentration and the kinetic constants K_m (Michaelis constant) and V_{max} (maximum velocity) but also on so-called immobilization effects. These effects are results of the following alterations of the enzyme by the immobilization process.

1.4.1. Changes in Enzyme Conformation

Conformation changes of the enzyme caused by immobilization usually decrease its affinity for the substrate (increase of K_m). Furthermore, a partial inactivation of all—or complete inactivation of part—of an enzyme molecule may occur (decrease of V_{max}). These two cases of a conformation-induced drop of V_{max} may be distinguished by measuring the activity of the resolubilized enzyme by titration of the active center with an irreversible inhibitor.

1.4.2. Changes in Microenvironment

Ionic, hydrophobic, or other interactions between the enzyme and the matrix (microenvironmental effects) may also result in changed K_m and V_{max} values. These essentially reversible effects are normally caused by variations in the dissociation equilibrium of charged groups of the active center. Once an enzyme has been insolubilized, however, it finds itself in a microenvironment that may be drastically different from that existing in a free solution. The new microenvironment may be a result of the physical and chemical character of the support matrix alone or it may result from interactions of the matrix with substrates or products involved in the enzymatic reaction.

1.5. Nonuniform Distribution

A nonuniform distribution of substrate and/or product between the enzyme matrix and the surrounding solution affects the measured (apparent) kinetic constants.

1.5.1. Diffusion Limitations

In biosensors, the biocatalyst and the signal transducer are spatially combined (i.e., the enzyme reaction proceeds in a layer separated from the measuring solution). The substrate molecules reach the membrane system of the biosensor by convective diffusion from the solution.

Diffusion limitations are observed in various degrees in all immobilized enzyme systems. This occurs because the substrate must diffuse from the bulk solution up to the surface of the immobilized enzyme prior to reaction. The rate of diffusion relative to enzyme reaction determines whether there are limitations on intrinsic enzyme kinetics. The rate at which the substrate passes over the insoluble particle affects the thickness of the diffusion film, which in turn determines the concentration of the substrate in the vicinity of the enzyme and hence the rate of the reaction.

Molecular weight of the substrate may also play a large role. Diffusion of large molecules will obviously be limited by steric interactions with the matrix. This is reflected by the fact that the relative activity of bound enzymes toward high-molecular-weight substrates has generally been found to be lower than that towards low-molecular-weight substances. This may, however, be an advantage in some cases, because the immobilized enzymes may be protected from attack by large inhibitors molecules.

1.6. Enzyme Immobilization for Acetylcholinesterase-Biosensor Construction

1.6.1. General Considerations

Acetylcholinesterases (AChEs) are enzymes that are present in both invertebrates and insects and participate in nervous impulse transmission processes. These enzymes hydrolyze the natural neurotransmitter acetylcholine (ACh) and are inhibited by determined substances, among them organophosphorus and carbamate pesticides.

Amperometric biosensors based in immobilized AChEs have been used for the detection of these pesticides in several matrices (e.g., standard solutions, water, food) and their functionality is based on enzyme inhibition that is proportional to the pesticide amount present in the sample or solution.

Recently, the screen-printed electrodes have become the most widely used devices for construction of sensitive biosensors and, depending on the working electrode mediator used, the working potential may be of variable values. Different enzyme immobilization procedures have been used for the fixation of AChE enzyme on the analytical electrode, including physical entrapment with glutaraldehyde and occlusion by polymerization in PVA with styrylpyridinium groups (PVA-SbQ).

In the method described here, 7,7,8,8-tetracyanoquinodimethano (TCNQ)-modified AChE-based biosensors will be constructed and used for detection of organophosphorus (OPs) insecticides. Immobilization procedure is based on polymerization with PVA-SbQ.

2. Materials

2.1. Preparation of the Screen-Printed Electrodes

1. DEK 248 printing Machine (DEK Printing Machines Ltd, www.dek.com).
2. PVC sheets.
3. Printing pastes: Electrodag® PF 410, Electrodag 423SS, and Electrodag 603SS (Acheson, Plymouth, UK).
4. Graphite T15 (Lonza, Switzerland).
5. Hydroxyl ethyl cellulose (HEC) (Fluka, Germany).
6. TCNQ: 7,7,8,8 Tetracyanoquinodimethane (Aldrich-Sigma, France).
7. Acetone: sodium dodecyl sulfate (SDS).
8. PVA-SbQ: polyvinylalcohol bearing styryl pyridinium groups (Toyo Gosei, Japan www.toyogosei.net).
9. AChE: acetylcholinesterases from electric eel (Sigma-Aldrich, www.sigma aldrich.com).

2.2. Tests of the Prepared Biosensor

1. 641 Metrohm detector (Metrohm, Zurich, Switzerland).
2. Flat bed recorder BD40 (Kipp and Zonen, the Netherlands).
3. Phosphate buffer solution (PBS) containing 0.05 *M* KCl, pH 7.5.
4. ATChCl : acetylthiocholine chloride (Sigma-Aldrich).
5. Paraoxon ethyl and dichlorvos (Dr. Ehrenstorfer, www.ehrenstorfer.de).

2.3. Solutions

1. Stock and working pesticide solutions. The pesticides paraoxon ethyl and dichlorvos will be used in this experiment. Prepare a 10^{-2} *M* stock solution by dissolving in acetone; use distilled water in order to prepare a more diluted working solution (*see* **Note 1**).
2. PBS containing KCl 0.05 *M*, pH 7.5: prepare the PBS solution by dissolving 18.2 g Na_2HPO_4, 3.6 g KH_2PO_4, and 3.0 g KCl in 1 L of distilled water. After solution homogenization, measure and adjust the pH to 7.5 with diluted NaOH (*see* **Note 2**).
3. Stock and working enzyme solutions: a commercially available AChE from electric eel [(ee)AChE] will be used. Prepare the AChE stock solution by dissolving the powder enzyme in an adequate 0.9% NaCl solution (*see* **Notes 3** and **4**). Working solutions can be prepared by dilution of the stock solution with PBS

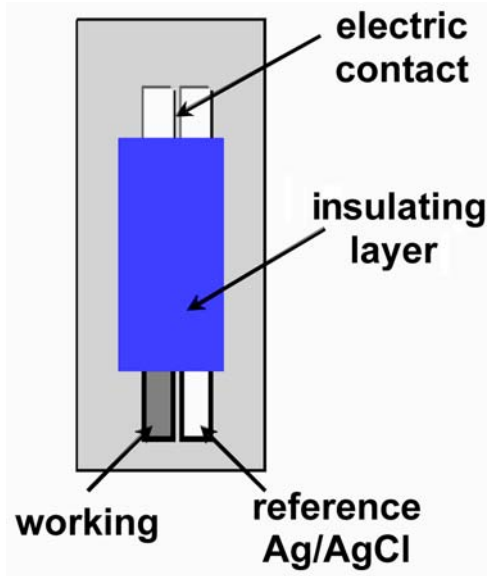

Fig. 2. Two-channel screen-printed electrodes used for AChE-biosensor construction.

and enzyme activity may be determined with these solutions by employing the Ellman method *(9)*.

3. Methods

3.1. Preparation of the Screen-Printed Electrodes

A single-channel two-electrode sensing strip (7 mm × 44 mm × 0.5 mm) will be used in this work. **Figure 2** presents a two-channel sensor strip, prepared as follows *(10,11)*:

1. Use PVC supports to prepare the strips with a DEK 248 screen-printer (DEK).
2. Print the conducting silver track (Electrodag PF410, Acheson) that forms the electrical connections between reference and working electrodes.
3. Print the graphite layer (Electrodag 423SS, Acheson) over the silver tracking to prevent contact between the tracking and applied analyte solution.
4. Print the silver/silver chloride ink (Electrodag 603SS, Acheson) reference electrode over the graphite layer on alternative silver tracks.
5. Print the working electrode prepared by mixing graphite T15 (Lonza) with TCNQ-mediator and hydroxyethyl-cellulose (HEC) over the remaining graphite-coated silver tracks. Prepare the graphite-TCNQ-HEC mixture by diluting 1 g graphite in 75 mL acetone followed by vacuum-evaporation for 30 min; then mix 0.9 g of the resulting powder with 5.8 mL of a 3% (w/v) HEC aqueous solution.
6. After homogenization, deposit the resulting paste by printing it on the working electrode. After applying the mediator layer, dry the electrodes at 60°C before enzyme immobilization for a minimum of 2 d.

7. Finally, deposit 2 µL of the sensitive paste, consisting of a mixture of determined volumes of PVA-SbQ and AChE solution (final amounts: 30% [v/v] for PVA-SbQ and 1 U/mL for AChE) (*see* **Note 5**).

3.2. Tests of the Prepared Biosensor

3.2.1. Electrochemical Measurements

Acetylthiocholine chloride (ATChCl) will be used as the substrate. Carry out the electrochemical measurements as follows:

1. Make chronoamperometric measurements at room temperature in PBS by using a potentiostat, imposing a constant potential of 100 mV between screen-printed working and reference (Ag/AgCl) electrodes (*see* **Note 6**).
2. To perform the measurements, immerse the biosensor in a cell containing 5 mL PBS under magnetic stirring and measure the stabilized electrical current (nA) (*see* **Notes 6** and **7**).
3. Immerse the biosensor in the same cell containing 4950 µL of PBS under magnetic stirring, and add 50 µL of 0.1 *M* ATChCl solution (substrate final concentration: 10^{-3} *M*). Register the stabilized electrical current (nA) after substrate addition.

3.2.2. Optimization of Substrate Concentration

1. Prepare solutions of varied concentrations (0.1–10 m*M*) in PBS and test the biosensor response (current, in nA) according to the previously described procedure. Construct a calibration curve of substrate concentration (mM) vs current (nA), and select the minimum substrate concentration to furnish a current intensity of approx 100 nA (*see* **Note 8**).

3.2.3. Stabilization of the Biosensor

After selecting the best ATCh concentration, repeat the electrochemical measurement during specific enzyme–substrate reaction at least 10 times in order to verify the biosensor stability (*see* **Note 9**).

3.2.4. Inhibition Assays

Paraoxon ethyl and dichlorvos of different concentrations will be tested. Carry out the inhibition assays as follows:

1. Record the stabilized current intensity after electrochemical measurement with the optimized substrate solution (S_o).
2. Carefully wash the biosensor with PBS and immerse it in the pesticide solution. Let it incubate in the pesticide solution for 10 min.
3. Carefully wash the biosensor with PBS and carry out another electrochemical measurement with the same substrate solution. Record the stabilized current intensity (*nA*) (S_1).
4. Calculate the percentage of relative inhibition (RI [%]), according the following equation:

$$\text{RI (\%)} = [1 - (S_1/S_o)] \times 100$$

5. Repeat the inhibition assay with the two pesticides at different concentrations and prepare two inhibition curves (pesticide concentration vs relative inhibition). Observe and compare the curves shapes (*see* **Note 10**).

3.2.5 Determination of the Detection Limits

Calculate and compare the limits of detection (LOD) for both OP pesticides. This LOD value is calculated with basis on 10% inhibition of the acetylcholinesterase, according to the inhibition assay previously described. Express the results in millimolarity (m*M* and parts per billion (ppb).

4. Notes

1. Pesticide solutions prepared by dilution with distilled water are very unstable. Working solutions can be also prepared by diluting the stock ones in PBS. These working solutions must be used and discharged at the end of the day.
2. Phosphate buffer may be prepared by using Sörensen's tables, but it is important to add the KCl (concentration: 0.05–1.0 *M*) in order to facilitate the electroconductivity of the solution between reference and working electrode.
3. AChE stock solutions are more stable in 0.9% NaCl. This solution must be kept at –4°C during storage. Use buffer solution only to dilute it before performing electrochemical assays.
4. AChEs from other sources (e.g., bovine erythrocytes, human erythrocytes) and butyrylcholinesterases (e.g., from horse and human serums) can also be used in this study. Also, genetically modified cholinesterases have proved to be very sensitive to several pesticide inhibitors ***(10,11)***.
5. The Ellman method ***(9,10)*** has to be applied in order to determine the specific activity (in activity unit [U]/mL) of the AChE working solution. This must be performed prior to enzyme immobilization in order to calculate the exact amount of the enzyme solution to be added to the PVA-SbQ in order to obtain an enzymatic charge of 1 U/electrode after immobilization
6. Chronoamperometry is performed by applying a potential of 100 mV vs silver/silver chloride electrode, and the current is recorded with time. Current stabilization takes less than 5 min with this system. If the stabilization takes an extremely long time and results in poor current (CV > 10 %) then the obtained electrode should be changed. A new immobilization procedure, with careful homogenization of the enzymatic paste, will need to be performed.
7. The current intensity obtained after signal stabilization means the biosensor capacity. This is the practical baseline for the followed measurements.
8. This initial current value is adequate to measure and detect lower pesticide contents. Higher values may decrease the biosensor sensitivity because minor variations in current signal may be factored into the noise scale.
9. A curve of type number of determinations vs current intensity (nA) can be constructed and the coefficient of variation (%) must be determined.
10. From the complexity of the mechanisms and kinetics of AChE inhibition it is evident that the calibration curves will not have a simple linear correlation for some pesticides, but acceptable linear correlations can be obtained in a restricted region of the curve. This can be considered the practical working calibration range of the biosensor.

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22

Immobilization of Enzymes for Use in Organic Media

Patrick Adlercreutz

Summary

When enzymes are to be used in organic media, noncovalent immobilization methods are often used because enzymes in general are insoluble in those media. Usually, immobilization increases the observed catalytic activity drastically as compared with nonimmobilized enzyme powder. The main reason is that immobilization spreads the enzyme on a relatively large area, which facilitates the mass transfer of substrates and products. The morphology of the support material is thus of crucial importance. In many applications, a monolayer of enzyme on the support provides catalysts with good activity. In general, the enzyme is first present in an aqueous solution and water is then removed using a drying procedure. Simple and effective methods for the adsorption of lipase on porous polypropylene and deposition of α-chymotrypsin on Celite® are presented in this chapter.

Key Words: Adsorption; α-chymotrypsin; Celite; deposition; lipase; porous polymer.

1. Introduction

As a result of intense research over the past two decades, it is now generally realized that many enzymes express catalytic activity in organic media, and many useful applications in synthesis have therefore been presented ***(1)***. In this chapter "organic media" is defined as media consisting predominantly of organic solvents or other organic substances. Solvent free media, in which the substrates to be converted function as solvents as well, constitute an important example. The organic media contain small amounts of water, which the enzymes need in order to express catalytic activity. Ionic liquids and supercritical fluids have also been used as media for enzymatic reactions. In these applications, they share several characteristics with organic media, mainly because of the low water content in all these media. Immobilization of enzymes for use in ionic liquids and supercritical fluids is treated separately in Chapters 23 and 24.

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Proteins are soluble in very few organic solvents ***(2)***. This means that in the majority of the organic media the enzymes are insoluble. It is therefore unnecessary to immobilize the enzymes in order to make it easier to separate them from the reaction mixture after the reaction. In many small-scale reactions, enzyme powders have been used as catalysts. However, immobilization is usually beneficial in order to improve the properties of the enzyme preparation. It is therefore quite common to immobilize enzymes by adsorption or deposition on porous supports before using them in organic media. The support material can be chosen so that it has suitable properties to be used in the intended reactor. In mechanically stirred reactors the materials should be resistant to shear forces, whereas in packed bed reactors the compressibility is more important because of its influence on the pressure drop of the reactor. In general, immobilization of the enzyme on a suitable support increases the observed catalytic activity as a result of effective dispersion of the enzyme on the surface of the support ***(3)***.

Enzyme immobilization methods for organic media are generally quite simple. An aqueous solution of the enzyme is mixed with the support, and the water is removed so that the enzyme is deposited on the surface of the support. The pH value of this solution is important. It has been said the enzyme "remembers" the pH of the last aqueous solution in which it was dissolved ***(4)***. The ionization state of the enzyme will remain essentially unchanged—unless acids or bases are present in the reactant solution—once the water has been removed and the enzyme preparation is put into an organic medium . Usually the aqueous solution used for enzyme immobilization is thus buffered to a pH value close to the pH optimum of the enzyme.

In some cases, enzymes adsorb spontaneously on the support. For example, it is possible to adsorb lipases efficiently on hydrophobic supports, such as porous polypropene. In this case, it is practical to use a rather large volume of aqueous solution in contact with the support. Lipases and other hydrophobic substances adsorb on the support whereas other substances remain in the solution. After complete adsorption, the immobilized enzyme is recovered by filtration. In this method, polar impurities in the enzyme powder used to prepare the solution are efficiently removed and thus immobilization simultaneously constitutes an enzyme purification step.

Not all enzymes are suitable for immobilization by adsorption. Some enzymes are adsorbed too strongly and thereby lose activity whereas others are not adsorbed efficiently enough. For the latter case, "deposition" constitutes a useful alternative method. Here, the enzyme is dissolved in a smaller volume of aqueous buffer, which is mixed with the support and followed by drying of the complete mixture. In this case, everything present in the solution is deposited on the support. This procedure has been found useful for a wide range of enzymes on a wide range of supports. Celite® is a typical support used in the deposition method.

The removal of water is a critical step in the immobilization processes for organic media. Most commonly, this is achieved by drying or freeze drying, but in some cases these procedures cause partial inactivation of the enzyme. Removal of water by treatment with water-miscible solvents such as propanol has been presented as a method to prepare highly active immobilized enzyme preparations for organic media ***(5)***.

Whatever immobilization method is used, the specific activity of an enzyme that has been immobilized on a support material and used in an organic medium is influenced by the nature of the support and by the enzyme loading (amount of enzyme per amount of support material). At low-enzyme loadings, low-specific activities are often observed as a result of inactivation of the enzyme ***(6)***. Presumably, an interaction between the enzyme and the support that is too strong is the main reason, and the chemical nature of the support is thus of key importance. In addition, the morphology of the support has a large influence ***(7)***. The specific surface area is a key parameter and it should be considered in relation to the enzyme loading. It has been observed that maximal specific activity is often obtained when the enzyme forms a monolayer on the surface of the support. At enzyme loadings too low to form a monolayer, the specific activity can often be increased by adding another protein (e.g., albumin) or another polymer (e.g., polyethylene glycol [PEG]) to protect the enzyme from inactivation ***(6)***. Under other conditions, mass transfer limitations can reduce the observed specific activity of the immobilized enzyme. This is common at high-enzyme loading and/or large particle size. Finally, the pore size of the support is of vital importance. The pores should be large enough to permit penetration by the enzyme during the immobilization procedure and diffusion of substrates and products during the reaction. In a lipase-catalyzed reaction, it was observed that maximal specific activity was obtained at a mean pore diameter of about 100 nm ***(8)***.

Simple protocols for adsorption of enzymes on hydrophobic supports and deposition of enzymes on Celite are described in **Subheading 3.** A third method of importance uses adsorption on ion exchange materials. Quite efficient adsorption is achieved when the ion exchanger and the enzyme are oppositely charged. Finally, it should be mentioned that covalent attachment of enzymes on supports is sometimes used in organic media, particularly when the reaction mixtures contain surfactants or other substances that can cause solubilization of the enzyme in the medium. Many of the methods described for covalent immobilization for aqueous media can be used in organic media as well.

2. Materials

2.1. Adsorption of Lipase on Porous Polypropene

1. Porous polypropene, Accurel MP1000 (Membrana GmbH, Obernburg, Germany; *see* **Note 1**).
2. 4.5-mL Glass vials with caps.
3. 20-m*M* Na-phosphate buffer, pH 6.0 (*see* **Note 2**).
4. 95% Ethanol.
5. Lipase powder. Lipases immobilized using this procedure include those from *Aspergillus niger*, *Candida rugosa*, *Penicillium roquefortii*, *Pseudomonas fluorescence*, *Rhizopus oryzae*, and *Thermomyces lanuginosus* ***(9)***.
6. End-over-end mixer.
7. Sintered-glass filter funnel, Buchner flask, and connection to suction pump.
8. Vacuum desiccator.
9. Vacuum pump (to give a pressure of 20 mbar or lower).

2.2. Deposition of α-Chymotrypsin on Celite

1. Celite, BDH (*see* **Note 3**).
2. α-Chymotrysin (Type II, Sigma, St. Louis, MO).
3. 95% Ethanol.
4. Deionized water.
5. Nitric acid.
6. Overhead stirrer.
7. 50 m*M* Tris-HCl buffer, pH 7.8 (*see* **Note 4)**.
8. Vacuum desiccator.
9. Vacuum pump (to give a pressure of 20 mbar or lower).

3. Methods

3.1. Adsorption of Lipase on Porous Polypropene

This procedure has been used for the immobilization of several different lipases ***(9)***. The immobilized preparations obtained are useful for a wide range of reaction including ester synthesis ***(3)***, racemate resolution ***(10)***, and lipid conversions ***(11)***.

1. Dissolve the lipase in 20 m*M* phosphate buffer, pH 6.0 (*see* **Note 5**). A suitable concentration is 0.1 to 1.0 mg lipase protein/mL.
2. Mix 50 mg Accurel MP1000 with 150 µL of 95% ethanol in a 4.5 mL capped vial.
3. Fill the glass vial completely with enzyme solution.
4. Incubate the vial on an end-over-end mixer at room temperature for at least 3 h (*see* **Note 6**).
5. Separate the immobilized preparation from the remaining solution by filtration. Wash the immobilized preparation briefly with the immobilization buffer (*see* **Note 7**).
6. Dry the immobilized preparation at reduced pressure in a vacuum desiccator overnight (*see* **Note 8**).

3.2. Deposition of α-Chymotrypsin on Celite

This procedure is a slightly modified version of a previously published method used to prepare Celite-immobilized α-chymotrypsin ***(12)***. Virtually the same method has been used for the immobilization of several other enzymes, including other proteases ***(13)***, lipases ***(14)***, oxidoreductases ***(15)***, and lyases ***(16)***. It is often very useful when trying a new enzyme in organic media.

1. Wash 50 g of Celite twice with 500 mL 95% ethanol each followed by 1500 mL deionized water each time for 15 times. After each washing, the liquid is decanted thus removing fines.
2. Dry the Celite at 80°C for 2.5 h.
3. Add 7 g of the washed Celite to a round flask containing 50 mL concentrated nitric acid. Stir the mixture with an overhead stirrer at 90°C for 4 h. Avoid grinding the particles (*see* **Note 9**).
4. Wash the Celite ten times with 100 mL deinonized water.
5. Dry the Celite at 80°C for 2.5 h.

6. Dissolve 180 mg α-chymotrypsin in 6 mL 50 m*M* Tris-HCl buffer, pH 7.8 (*see* **Note 10**).
7. Add the α-chymotrypsin solution to 6 g of the acid washed Celite. Mix thoroughly (*see* **Note 11**).
8. Spread out the preparation on the bottom of a wide beaker, which is put in a vacuum desiccator. Dry under vacuum overnight (*see* **Note 8**)

4. Notes

1. Different types of porous hydrophobic materials are useful for enzyme adsorption. Porous polypropene has been used extensively. The activity of the immobilized enzyme varies somewhat depending on the characteristics of the support. The porous polypropene Accurel EP-100 from Akzo has provided immobilized preparations with high activities in many studies, but this material is no longer available. However, the new material Accurel MP1000 available from Membrana GmbH gives similar results.
2. In order to get maximal catalytic activity, the buffer used should have a pH value close to the pH optimum of the enzyme. The buffer mentioned here, 20 mM Na-phosphate buffer, pH 6.0, has been used successfully for a wide range of lipases.
3. Celite of different types can be used. Depending on the chacteristics, differences in catalytic activity are obtained. Celite intended for gas chromatography from BDH (available from VWR) with mesh size 30 to 80 or 80 to 120 has been widely used, but there are many other useful types commercially available.
4. The buffer should be chosen to fit the enzyme to be immobilized (*see* **Note 2**). Both the type of buffer and its concentration will have an influence on the catalytic activity of the immobilized preparation.
5. If the lipase powder contains substances which are insoluble in water, those can be removed by centrifugation or filtration.
6. The adsorption process can be followed by measuring protein content and enzyme activity in the solution. Lipase adsorption is usually complete after 3 h. Often adsorption has been carried out overnight with similar results.
7. A small amount of aqueous buffer will remain in the immobilized preparation after filtration. During drying, the buffer salts will be deposited on the support surface. Because the buffer salts can influence the activity of the immobilized preparation, it is advisable to standardize the immobilization procedure to get reproducable results.
8. The drying process can be monitored gravimetrically or by water analysis.
9. Different types of Celite vary concerning the need for acid washing. By using the procedure described, immobilized preparations with high and reproducible catalytic activity have been obtained.
10. Make the solution just before using it. Prolonged incubation can lead to autolysis and reduced catalytic activity.
11. Here, 1 mL of aqueous solution is used with 1 g of Celite. If it is difficult to achieve uniform wetting of the Celite with these proportions, the volume of the aqueous solution can be increased (by dilution with water).

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23

Immobilization of Enzymes for Use in Ionic Liquids

Pedro Lozano, Teresa de Diego, and José L. Iborra

Summary

The use of ionic liquids (ILs) as nonconventional reaction media in enzyme catalyzation has gained increasing attention for developing green chemical processes because of the physical and chemical characteristics of ILs. Some have been shown to be by far the best nonaqueous media for enzyme-catalyzed reactions, because the enzymes display a high level of activity and stereoselectivity in synthesizing many different substrates, as well as by their excellent ability to stabilize enzymes toward re-use. The different operational strategies used for enzymatic processes in ILs are analyzed. Protocols to synthesize different fine chemical products, such as aspartame, butyl butyrate, R-2-pentyl propionate, *N*-acetyllactosamine, and polyester are described in detail, including analytical techniques. A detailed description of the procedure for recovering and cleaning of ILs to be re-used is also included, as well as notes containing special cautions.

Key Words: Biotransformations; enzyme stability; ionic liquids; green chemistry; lipases; nonconventional media.

1. Introduction

Enzymatic reactions based on ionic liquids (ILs) are promising alternatives to organic solvents in the biotechnological research area known as "biocatalysis in nonconventional media." Organic solvents are usually volatile liquids that evaporate into the atmosphere with detrimental effects on the environment and human health. Therefore, it is necessary to develop new alternatives for their enzyme-catalyzed biotransformation in friendly environmentally reaction media ***(1)***.

What are ILs? They are simply liquids composed entirely of ions at or close to room temperature. Using sodium chloride as an example, molten sodium chloride (up 800°C) is an IL, whereas an aqueous solution of this salt is an ionic solution ***(2)***. Typical room temperature ionic liquids (RTILs) are based on organic cations (e.g., 1,3-dialkylimidazolium, *N*-alkylpyridinium, tetraalkylammonium, and tetraalkylphosphonium) paired with a variety of anions that have a strongly delo-

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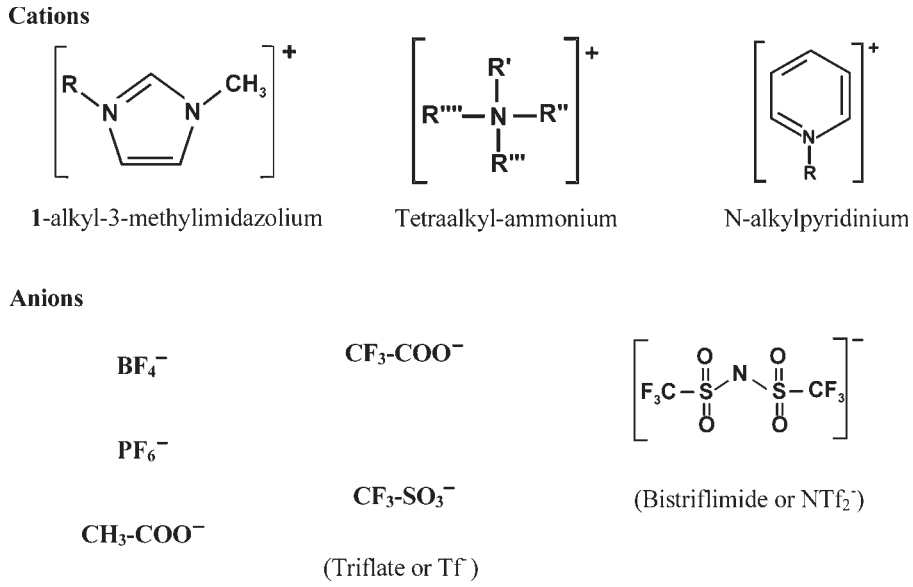

Fig. 1. Structures of typical ions of ILs used for enzymatic reactions.

calized negative charge (e.g., BF_4^-, PF_6^-, SbF_6^-, triflate, bistriflimide; *see* **Fig. 1**), resulting in colorless, low-viscosity, and easily manipulable materials with very interesting properties as solvents. These properties includes negligible vapor pressure (they do not therefore evaporate), excellent thermal stability (up 300°C in many cases), a high ability to dissolve a wide range of organic and inorganic compounds, including gases (e.g., H_2, CO_2, CO,), nonflammable nature, high conductivity, and a large electrochemical window. Moreover, their polarities, hydrophilicities/hydrophobicities and solvent miscibility can be tuned by selecting the appropriate cation and anion. ILs can be designed to be miscible or immiscible with water or some organic solvents (e.g., hexane, benzene, ether, *i*-PrOH), making them more useful for recovering products from the reaction mixture (**Table 1**) ***(3)***. Spectroscopic measurements of solvatochromic and fluorescence probe molecules in ILs suggest that these solvents have a polarity comparable with that of the lower alcohols (e.g., methanol, ethanol). All of these properties, including the fact that they are re-usable and recyclable, allow ILs to be considered ideal solvents for green chemistry.

Enzymatic transformations in ILs have only recently been considered (first paper published in 2000 ***(4)*** and a wide number of applications have been tested. Some ILs have been shown to be by far the best nonaqueous media for enzyme-catalyzed reactions, because the enzymes display a high level of activity and stereoselectivity in synthesizing many different compounds (e.g., aspartame) ***(4)***, aliphatic and aromatic esters ***(5–8)***, aminoacid esters ***(9–11)***, quiral esters by kinetic resolution of racemic alcohols ***(12–20,25)***, carbohydrate esters ***(21,22)***, and polymers ***(23,24)***.

Table 1
Physical Properties of Some Ionic Liquids

Cation	Anion	T_{melt} (°C)	Density at 20°C (g/mL)	Viscosity at 20°C (cSt)	Solubility				
					H_2O	AcN	*i*PrOH	Hex	Tol.
$Mmim^+$	$MeSO_4^-$	–	1.33	70					
$Emim^+$	BF_4^-	15	1.34	113	+	+	–	–	–
$Bmim^+$	BF_4^-	–71	1.21	120	+	+	–	–	–
$Hmim^+$	BF_4^-	–82	1.15	195	±	+	+	–	–
$Emim^+$	PF_6^-	60	Solid	Solid	+	+	–	–	–
$Bmim^+$	PF_6^-	12	1.37	281	–	+	–	–	–
$Hmim^+$	PF_6^-	–74	1.29	548	–	+	–	–	–
$Emim^+$	NTf_2^-	–3	1.52	145	–	+	–	–	–
$Bmim^+$	NTf_2^-	–16	1.43	256	–	+	–	–	–

+, Totally miscible; –, immiscible; ±, partially miscible (*see* **Note 2)**.

From an overall point of view, three different operational strategies have been applied for enzymatic processes in ILs: (1) pure ILs in monophasic systems; (2) water–IL mixtures in monophasic systems; and (3) pure ILs in biphasic systems.

1. As pure solvents in monophasic systems, different water-immiscible ILs (e.g., 1-butyl-3-methylimidazolium hexafluorophosphate [$bmim^+$ PF_6^-], 1-ethyl-3-methylimidazolium *bis*[trifluoromethane] sulfonylamide [$emim^+$ NTf_2^-]) have been assayed as reaction media for biocatalytic reactions, by using both free or immobilized enzymes with excellent results ***(4–25)***. On the contrary and in all cases, no enzymatic activity was observed when anhydrous water-miscible ILs were used as reaction media. Furthermore, an additional new concept should be discussed. Classically, immobilized enzymes obtained by chemical or physical attachment onto solid supports have been considered advantageous over free enzyme molecules because they facilitate the recovery and reuse of the biocatalyst, allowing continuous processes to be designed. However, in the case of water-immiscible ILs, free enzyme molecules suspended in these media behaved as anchored or immobilized biocatalysts, because they cannot be separated by liquid–liquid extraction (i.e., with buffer or aqueous solutions) ***(6,7,9,12–14,18,19)***. To eliminate protein molecules from ILs, it is necessary filter the enzyme–IL solution through ultrafiltration membranes with a cut-off lower than the molecular weight of the enzyme ***(22)***. ILs form a strong ionic matrix and the added enzyme molecules could be considered as included rather than dissolved in the media (*see* **Fig. 2**). ILs therefore should be regarded as being liquid enzyme immobilization supports, rather than reaction media ***(6)***. In this way, after an enzymatic transformation process in ILs, the products can be recovered by liquid–liquid extraction with solvents ***(5–8,12,13,17,19–24)***, or by reduced pressure ***(14)***, pervaporation ***(18)***, or by extraction with supercritical carbon dioxide ($scCO_2$) ***(15,25)***, enabling the enzyme-IL system to be reused in consecutive operation

cycles. Several authors have described the excellent stability of free enzymes in water-immiscible ILs towards reuse (up to 2300-fold half-life time with respect to classical organic solvents) ***(6,9,19,24)***. In this context, even if enzyme derivatives immobilized onto solid supports (e.g., Novozyme®, a commercial lipase derivative from Novo Nordisk) have been widely assayed in pure IL systems, the classical advantage that they have over free enzymes (e.g., the protective effect of the solid support against denaturative conditions) could be in doubt, because of the exceptional stabilization of free enzymes towards continuous reuse provided by ILs (e.g., free CALB in $bmim^+$ PF_6^- exhibited a half-life time 2300 times greater than that observed in hexane at 50°C) ***(6)***. Also, it has been demonstrated that both free and immobilized *Candida antarctica* lipase B suspended in $bmim^+$ NTf_2^- showed similar profiles of activity decay in continuous operation under anhydrous and extremely harsh conditions, such as $scCO_2$ at 150°C and 10 MPa ***(25)***. In spite of these facts and because of the relative higher viscosity of ILs compared with water or organic solvents, novel materials, based on high-density metal oxides (e.g., WO_3/TiO_2, WO_3/ZrO_2) have been assayed as suitable supports for lipase immobilization by adsorption. Such enzyme derivatives have been used in ILs systems with excellent results as regards activity and enantioselectivity ***(17)***.

2. Water-miscible ILs (e.g., *N*-ethylpyridinium trifluoroacetate, 1,3-dimethylimidazolium methyl sulfate) have been described as suitable reaction media for enzymatic transformations when they were assayed in monophasic mixtures with water or aqueous solutions. In these conditions, IL–water mixtures should be considered as ionic solutions of organic salts. Several enzymes, such as subtilisin, lipase, and β-galactosidase, were successfully assayed in these systems for the resolution of aminoacid esters, regioselective acetylation of glucose and transglycosylation of lactose, respectively ***(11,21,22)***. Hydrolytic enzymes exhibited behavior in aqueous solution of water-miscible ILs similar to that observed in classical water miscible organic solvents (e.g., *N*,*N*-dimethylformamide, acetonitrile), characterized by maximal synthetic activity at a high water content, gradually decreasing until full enzyme deactivation at a low water content.
3. The third strategy assayed for enzyme-catalyzed reactions in ILs involves the use of these solvents as liquid or solid enzyme immobilization supports in nonaqueous biphasic systems ***(15,16,25)***. In such cases, the ability of ILs to retain free enzyme as a homogeneous phase (catalytic phase), whereas substrates and products reside largely in another phase (extractive phase), have successfully been assayed for lipase-catalyzed kinetic resolution in nonaqueous environments (e.g., organic solvents and $scCO_2$). For the classical concept of immobilized enzymes, ILs with melting points higher than room temperature [e.g., 1-(3'-phenylpropyl)-3-methylimidazolonium hexafluorophosphate melts at 53°C] can also be used to easily obtain solid enzyme-IL particles then to be apply in organic reaction media ***(16)***.

2. Materials

2.1. Ionic Liquids (see Fig. 1 and Notes 1–3)

1. 1-Butyl-3-methylimidzolium hexafluorophosphate ($bmim^+$ PF_6^-; Solvent Innovation GmbH).

2. 1-Ethyl-3-methylimidzolium bis(trifluoromethane)sulfonylamide (emim$^+$ NTf_2^-; Sigma Aldrich Chemical Co.).
3. 1-Buthyl-3-methylimidzolium bis(trifluoromethane)sulfonylamide (bmim$^+$ NTf_2^-; Merck).
4. 1, 3-Dimethylimidazolium methylsulfate (mmim$^+$ $MeSO_4^-$; Merck).
5. Themoslysins substrate solution: dissolve 100 mmol of Cbz-L-aspartate and 500 mmol of L-phenylalanine ethyl ester into the wet IL, and shake the mixture mechanically at room temperature until a clear solution is obtained
6. The mobile phase for Z-aspartame synthesis will consist of acetonitrile/water (60/40 [v/v]), containing the aqueous fraction 0.2% (w/v) triethylamine, which is adjusted to pH 2.5 with orthophosphoric acid.
7. CAL-B substrate solution: for the butyl butyrate synthesis. Dissolve 90 µL (0.71 mmol) of vinyl butyrate and 330 µL (3.63 mmol) of 1-butanol in 900 µL of dry emim$^+$ NTf_2^- into a screw-capped test tube. A clear solution must be obtained.
8. Reaction mixture for the *CALB*-catalyzed kinetic resolution of 2-pentanol: dissolve 138 µL (1.25 mmol) of vinyl propionate and 138 µL (1.25 mmol) of *rac*-2-pentanol in 900 µL of dry bmim$^+$ NTf_2^- in a screw-capped test tube. A clear solution must be obtained.
9. Reaction mixture for the β-galactosidase-catalyzed *N*-acetyllactosamine synthesis Add 2.5 mL of mmim$^+$ $MeSO_4^-$ to a screw-capped test tube containing 4 mL of 1.5 *M N*-acetylglucosamine in 0.1 *M* potassium phosphate buffer, pH 7.3, and 2.5 mL of 250 m*M* lactose in 0.1 *M* potassium phosphate buffer, pH 7.3. Shake the resulting mixture mechanically at room temperature until a clear solution is obtained.
10. Reaction mixture for the polyester synthesis. Dissolve separately 200 µmol of divinyl adipate and 200 µmol of 1,4-butanediol in screw-capped test tubes containing 1 mL of dry bmim$^+$ PF_6^-, respectively. Shake both mixtures mechanically for 30 min at 50°C to reach full solubilization of the monomers.

2.2. Enzymes

1. Thermolysin (EC 3.4.24.27; Sigma-Aldrich).
2. *Candida antarctica* lipase B (CALB; EC 3.1.1.3.; Novo Nordik).
3. *Pseudomonas cepacia* lipase (PS-C; EC 3.1.1.3.; Sigma).
4. *Bacillus licheniforms* subtilisin (Alcalase; EC 3.4.21.62; Novo Nordik).
5. β-Galactosidase (EC 3.2.1.23; Sigma).

3. Methods

3.1. Thermolysin-Catalyzed Z-Aspartame Synthesis in Bmim$^+$ PF_6^- (4)

1. Into a screw-capped test tube containing 5 mL of bmim$^+$ PF_6^-, add 200 µL of water and shake the mixture mechanically at room temperature until a clear solution is obtained (*see* **Note 4**).
2. Start the reaction by adding 50 µL of 1% (w/v) thermolysin solution in water (*see* **Fig. 3**).
3. Mix mechanically the reaction mixture (i.e., by a rotary disk shaker) for up to 50 h at 37°C to obtain full product conversion.

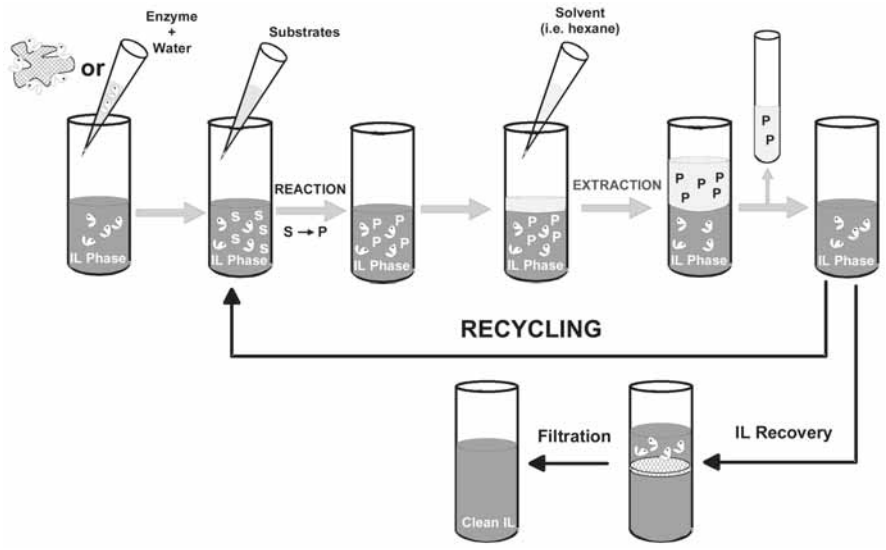

Fig. 2. Operational strategy for enzyme catalysis in monophasic ILs systems, including recycling and IL recovery processes.

4. To follow the enzyme reaction, 100 µL aliquots can be taken from the reaction mixture at appropriate times, and dissolved in 1.5 mL of mobile phase.
5. Analyze samples by high-performance liquid chromatography (HPLC) using a LiChrospher RP-18 column (25-cm length and 3.9-mm internal diameter, 5-µm particle size, and 10-nm pore size; Merck) in isocratic conditions at 1 mL/min flow rate. Elution profiles can be easily monitored at 257 nm by a DAD or UV detector.
6. To extract all product and remaining substrates, the full reaction mixture should be washed with water. Add a 10-fold excess water volume to the reaction mixture and shake vigorously for 15 min to carry out liquid–liquid extraction of the remaining substrates. Decant the aqueous phase. Remove the remaining water into the IL phase by vacuum evaporation, leading to precipitation of the product. Recover product crystals by filtration.
7. The resulting thermolysin–bmim$^+$ PF_6^- system can be reused by adding an aqueous solution of fresh substrates.

3.2. CALB-Catalyzed Butyl Butyrate Synthesis in Emim$^+$ NTf_2^- (6,7)

1. Start the reaction by adding 20 µL of 1 % (w/v) CALB solution in water (*see* **Fig. 4**).
2. Mechanically mix the reaction mixture (e.g., by a rotary disk shaker) and incubate at 40°C for up to 1 h to obtain full product conversion.
3. To follow the reaction, 20-µL aliquots can be taken from the reaction mixture at regular appropriate intervals of time and suspended in 1 mL of hexane (*see* **Note 5**). Shake the biphasic mixture obtained vigorously for 3 min to extract all substrates and products into the hexane phase. Then, mix 400 µL of hexane extract with 600 µL

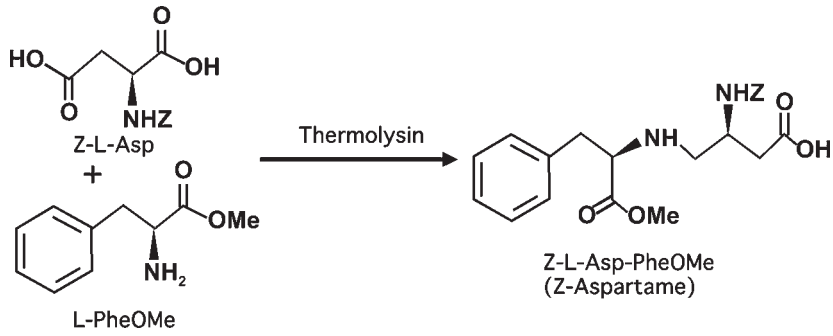

Fig. 3. Thermolysin-catalyzed carbobenzoxy-L-aspartyl-L-phenylalanyl methyl ester (Z-aspartame) synthesis from Z-L-aspartate and L-phenylalanine methyl ester.

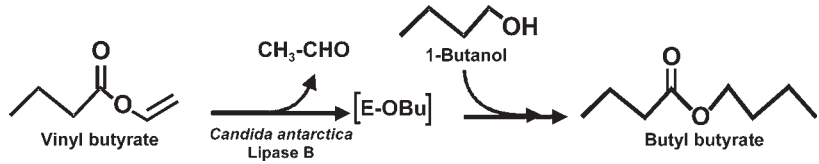

Fig. 4. Lipase-catalyzed butyl butyrate synthesis.

of 10 m*M* propyl acetate (internal standard) solution in hexane, and analyze 1 µL of the resulting solution chromatographically. Recover all the IL aliquots in a test tube for cleaning and reuse (*see* **Subheading 3.6.**).

4. Analysis of samples can be carried out by gas chromatography (GC) semicapillary Nukol column (15 m × 0.53 mm × 0.5 µm; Supelco) and a flame ionization detector. Chromatographic conditions are as follows: carrier gas (N_2) at 8 kPa (20 mL/min total flow); temperature program: 45°C, 4 min, 8°C/min, 133°C; split ratio, 5:1; detector, 220°C. Retention time of peaks are as follows: propyl acetate, 3.2 min; vinyl butyrate, 4.3 min; 1-butanol, 6.6 min; butyl butyrate, 7.7 min , and butyric acid, 13.5 min.
5. To extract all product and remaining substrates, the full reaction mixture should be washed with hexane. Add a 30-fold excess hexane volume to the reaction mixture and shake vigorously for 15 min to give liquid–liquid extraction of the product and remaining substrates.
6. The resulting CALB-emim$^+$ NTF_2^- system can be reused by adding fresh substrates, while the enzyme activity remain practically constant.

3.3. CALB-Catalyzed Kinetic Resolution of 2-Pentanol in bmim$^+$ NTf_2^- (20)

1. Start the reaction by adding 20 µL of 1 % (w/v) CALB solution in water (*see* **Fig. 5**).
2. The reaction mixture should be mechanically mixed (e.g., by a rotary disk shaker) and incubated at 60°C for up to 3 h to obtain full product conversion.

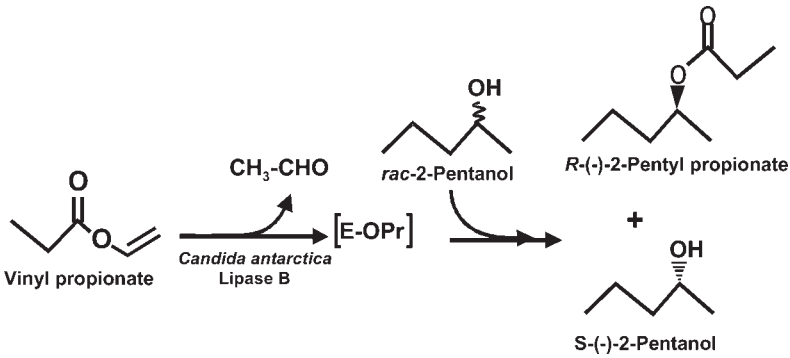

Fig. 5. Lipase-catalyzed kinetic resolution of *rac*-2-pentanol.

3. To follow the reaction, 20 µL aliquots should be taken from the reaction mixture at regular appropriate times and suspended in 1 mL of hexane (*see* **Note 5**). The resulting biphasic mixture is strongly shaken for 3 min to extract all substrates and products into the hexane phase. Finally, mix 200 µL of hexane extract with 100 µL of 300 m*M* ethyl propionate (internal standard) solution in hexane, and chromatographically analyze 1 µL of the resulting solution. Recover all the IL aliquots into a test tube for cleaning and reuse (*see* **Subheading 3.6.**).
4. Analysis of samples can be carried out by a GC β-DEX-120 capillary column (30 m × 0.25 mm × 0.25 µm; Supelco) and a flame ionization detector. Chromatographic conditions are as follows: carrier gas (He) at 1 MPa (205 mL/min total flow); temperature program: 60°C, 16 min, 15°C/min, 90°C; split ratio, 100:1; detector, 230°C. Retention times of the peaks are as follows: vinyl propionate, 6.3 min; ethyl propionate, 8.3 min; *R*-2-pentanol, 15.3 min; *S*-2-pentanol, 15.8 min; *R*-2-pentyl propionate, 22.2 min; *S*-2-pentyl propionate, 23.2; propionic acid, 25.1.
5. To extract all the product and remaining substrates, the reaction mixture should be washed with hexane. For this, add a 30-fold excess volume of hexane to the reaction mixture and shake vigorously for 15 min to give liquid–liquid extraction.
6. The resulting CALB-bmim$^+$ NTF_2^- system can be reused in several reaction cycles by adding fresh substrates, remaining practically constant the enzyme activity.

3.4. β*-Galactosidase-Catalyzed* N*-Acetyllactosamine Synthesis in mmim$^+$ $MeSO_4^-$ (22)*

1. Start the reaction by adding 1 mL of 1 % (w/v) β-galactosidase solution in 0.1 *M* potassium phosphate buffer, pH 7.3 (*see* **Fig. 6**).
2. Mix mechanically the reaction mixture (e.g., by a rotary disk shaker) at 25°C for up to 2 h to reach as least a half product yield (*see* **Note 6**).
3. To follow the enzyme reaction, take 100-µL aliquots from the reaction mixture at appropriate intervals of time, and heat to 100°C for 10 min to stop the reaction by thermal deactivation (*see* **Note 7**). Filter samples to remove proteins with a Minisart RC 4 system (Sartorius). The resulting clear solution is analyzed chromatographically.

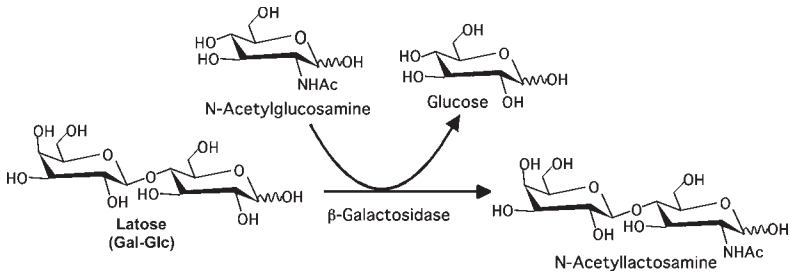

Fig. 6. β-Galactosidase-catalyzed *N*-acetyllactosamine synthesis by transglycosilation.

4. Analyze samples by HPLC using a Aminex HPX-87H column (25-cm length and 3.9-mm internal diameter, 5-µm particle size, and 10-nm pore size, BioRad) in isocratic conditions at 0.8 mL/min flow rate and 60°C. The mobile phase will consist of 6 m*M* sulfuric acid solution in water. Elution profiles can easily be monitored by using RI or UV (208 nm) detectors.

3.5. Immobilized CALB-Catalyzed Polyester Synthesis in $bmim^+ PF_6^-$ (23)

1. Mix both momomers solutions and start the reaction by adding Novozym 435 (*see* **Fig. 7** and **Note 8**).
2. Mix the reaction mixture mechanically (e.g., by a rotary disk shaker) at 50°C for less then 24 h to observe the precipitation of polymeric product.
3. Recover the polyester product from the IL reaction medium, and re-dissolve it with tetrahydrofuran (*see* **Note 9**). The molecular weight of re-dissolved polymeric products can be analyzed by gel permeation chromatography.
4. Analysis of samples can be carried out by a HPLC PL-gel MIXED E column (250-mm length and 4.6-mm internal diameter, 3-µm particle size; Polymer Laboratories) in isocratic conditions at 1 mL/min flow rate and 35°C, using tetrahydrofuran as mobile phase. Elution profiles can easily be monitored by RI detector (*see* **Note 10**).

3.6. Recovery and Cleaning of Ionic Liquids

1. For each of the proposed reactions, all the respective IL fractions, containing IL, enzymes, traces of reagents, and so forth, should be collected into a specifically labeled vessel.
2. For ILs immiscible with water, dissolve the IL fraction with a volume of acetonitrile (dilution 1:1, to reduce viscosity), and then filter the resulting solution through an ultrafiltration cell (Amicon, Millipore) equipped with a YM 3 membrane (cut-off 3000 Da.) at 6 bar (pressurized with N_2) and room temperature to separate the enzyme molecules (*see* **Fig. 2**).
3. Remove solvents by vacuum evaporation to concentrate the IL.
4. Add water in a 10-fold excess. Shake strongly the IL-water biphasic mixture for 3 min and decant, to extract any water-soluble compounds. Repeat this washing step twice.

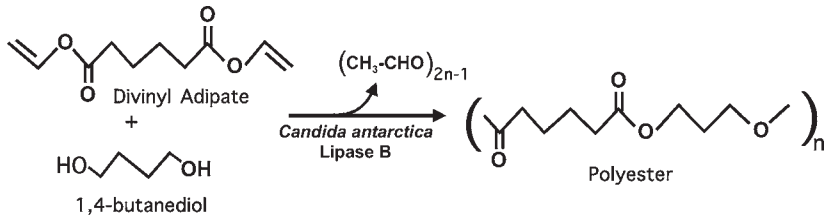

Fig. 7. Lipase-catalyzed polytransesterification of divinyl adipate and 1,4-butanediol.

5. Add hexane (HPLC degree) in a 10-fold excess. Shake strongly the IL-hexane biphasic mixture for 3 min and decant to extract hydrophobic compounds. Repeat this washing step twice. GC analysis of the hexane fraction can be used to check the cleaning degree, repeating the washing steps if necessary.
6. Recover the IL fraction and remove traces of solvents by vacuum evaporation at 50°C.
7. Keep the IL under dry conditions over P_2O_5 in a dessicator at room temperature.
8. For ILs miscible with water, the IL–H_2O fraction can be filtered directly in the same system. Remove water by vacuum evaporation to concentrate the IL.

4. Notes

1. Many references describe protocols to synthesize ionic liquids. A comprehensive review focused on the synthesis and purification of ILs is included in **ref. *2***.
2. Some start-up and chemical manufacturers of ILs are listed below:
   a. Solvent Innovation GmbH (*http://www.solvent-innovation.com*).
   b. Cytec (*http://www.cytec.com/*)
   c. Acros Chemical (*http://www.fisherchemicals.co.uk/*) markets ILs samples produced by QUILL (Ionic Liquids Laboratory, Queen's University of Belfast).
   d. Sigma Chemical Co. (*http://www.sigmaaldrich.com/*).
   e. Merck (*http://pb.merck.de/servlet/PB/menu/1122050/index.html*). This includes an excellent database, which allows a search for ILs according to their physical properties.
3. ILs are colorless and look like water. Traces of starting materials, oxidation products, thermal degradation products, halides, and so on are common impurities that could be present in commercial ILs. Purification of commercial ILs by treatment with carbon active and/or by washing with pure solvents is recommended to avoid undesired effects (*see* **Subheading 3.6.**). Keep ILs under dry conditions over P_2O_5 in a dessicator at room temperature.
4. Substrates are insoluble in dry $bmim^+$ PF_6^-, and it is necessary to saturate the IL with water beforehand. If an excessive amount of water is used, an undesirable wet IL–water biphasic system is formed. The aqueous phase must be decanted.
5. Before opening a screw-capped test tube, place it in an ice bath for 10 s to prevent the evaporation of substrates and products.
6. Increased concentrations of $mmim^+$ $MeSO_4^-$ produced a progressive decay in enzyme activity. A 25% (v/v) is sufficient to produce a decrease in Aw, which

has been associated with a reduction in the undesirable hydrolysis of substrate and synthetic product (*see* **Fig. 6**).

7. Care should be taken during thermal deactivation to avoid evaporation of the sample. The authors did not described the procedure used.
8. The authors did not indicate the amount of enzyme used; 50 mg of Novozym 435 may be appropriate.
9. The authors did not describe the procedure to extract the polymeric product separately from the IL and immobilized enzyme, respectively. Polyester product could be recovered by centrifugation of the reaction mixture at 2800 g for 30 min. The resulting precipitated fraction can be re-dissolved with tetrahydrofuran (e.g., 5 mL), and centrifuged again (e.g., 2800 g for 5 min) to separate immobilized enzyme particles. By this way, a clear solution of reaction products suitable for HPLC analysis can be obtained.
10. The authors proposed a system containing three gel permeation columns in series to achieve sufficient separation in the molecular weight range 500–30,000.

Acknowledgments

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Immobilization of Enzymes for Use in Supercritical Fluids

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Summary

Supercritical fluids (SCFs) are environmentally benign solvents that enable efficient ability to dissolve and/or transport many hydrophobic compounds. Because the solvent properties of SCFs can be adjusted by changing either the pressure or the temperature, they are widely used in a industrial extractive clean processes. For enzymatic biotransformation, several criteria must be considered to select an SCF as the reaction medium, such as the critical parameters or the safety and cost advantages. The importance of strategies for stabilizing enzymes toward SCFs, as well as the appropriate design of reactors is also discussed. Four different high-pressure reactors (i.e., stirred tank, packed bed, cross-flow membrane, and membrane with recirculation) used for enzymatic synthetic processes utilizing SCFs are described in detail. Protocols for operation with these reactors to carry out butyl butyrate synthesis or the kinetic resolution or rac-1-phenylethanol are described in detail, including procedures to obtain membranes with immobilized enzymes and a list of notes of special interest for researchers.

Key Words: Biotransformations; enzyme reactors; green chemistry; lipases; nonconventional media; supercritical fluids.

1. Introduction

Supercritical fluids (SCFs), in their role as nonconventional media for enzymatic reactions, are an interesting alternative for developing clean processes because of their interesting physical properties. SCFs are materials which operate at pressures and temperatures higher than their critical points (P_c and T_c) (*see* **Fig. 1**), having densities comparable to those of liquids, while their diffusivities and viscosities are similar to those of gases (***1***,***2***). These characteristics, which suit them as ideal solvents for use in extraction processes, also make them attractive as media for biocatalytic transformations, especially when reactions are limited by the rate of diffusion, rather than by any intrinsic kinetics (***3***,***4***). However the key feature of

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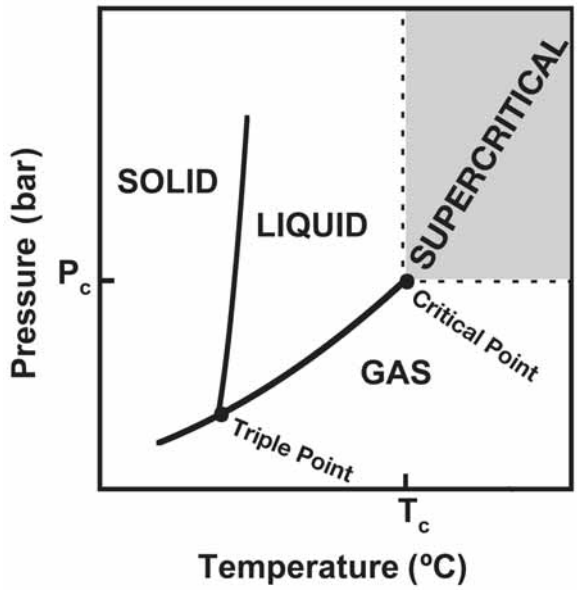

Fig. 1. Phase diagram of pure substances.

SCFs as solvents is their tunability with changes in pressure and temperature, for which reason they can be used to recover products from reagents and to modify the selectivity of reactions ***(5–7)***. The density of SCFs is highly sensitive to both temperature and pressure and so all their density-dependent solvent properties (e.g., dielectric constant, relative permittivity, Hildebrand solubility parameter) may be substantially modified by small changes in pressure or temperature. This provides a potential for controlling reactions by precipitation of a product, or for purification by the selective precipitation of products. Different pure substances can be used as supercritical fluids, some of them are used already in biocatalysis (**Table 1**). However, several criteria must be considered before selecting a given SCF as enzyme-reaction medium, including the critical parameters and safety and cost advantages. Supercritical carbon dioxide ($scCO_2$) is the most popular SCF because of its relatively low critical parameters, its low toxicity, and nonflammability; furthermore, it is chemically inert in most conditions, has excellent solvent properties for nonpolar solutes, and is considered as a green solvent ***(7)***. Carbon dioxide is clearly a "greenhouse gas," however, it is produced at an industrial scale as a by-product (e.g., ammonia plants). It is therefore cheap. Furthermore, at atmospheric pressure, CO_2 is gaseous, which means that simple depressurization is necessary to separate solutes from $scCO_2$, after which it can be pressurized for reuse as a solvent. The solvent power of $scCO_2$ can be modified by increasing the bulk density or by adding a modifier (e.g., MeOH or toluene). Cosolvents can therefore be used to increase or reduce polarity, or to enhance affinity for aromatic substances, although, of course, the more cosolvent that is added, the further $scCO_2$ moves away from being a truly green solvent ***(1)***. The most important limitation to

Table 1
Critical Parameters of Some SCFs *(2)*

Fluid	T_C (°C)	P_C (bar)	δ_c (g/mL)
Ethylene*	9.5	50.8	0.22
Xe	16.6	58.4	1.10
CHF_3*	25.9	46.9	0.52
CO_2*	31.3	73.8	0.47
Ethane*	32.3	48.8	0.20
N_2O	36.5	72.5	0.45
SF_6*	45.5	37.1	0.74
CCl_2F_2	111.8	40.7	0.56
Propane*	96.6	42.5	0.22
Butane*	152.0	37.5	0.23
Pentane	196.6	33.3	0.23

*, SCFs used in biocatalysis.

applying SCFs and then to any scaling-processes is the fact that the high-pressure equipment needed is quite costly.

Enzymes are not soluble in SCFs, and several free or immobilized enzymes (lipases, trypsin, chymotrypsin, penicillin acylase, and cholesterol oxidase) are used to catalyze chemical transformations (e.g., esterification, hydrolysis, alcoholysis) in SCFs ***(3–14)***. As in the case of nonaqueous solvents, the role of water in enzyme-catalyzed reactions in SCFs can be analyzed by the same rules as for organic solvents. Although, water is needed to maintain the active conformation of the enzyme, it can also act as a solvent for polar substances, if hydrolytic processes occur, and/or be consumed or produced *in situ* in the system, if esterification occurs. The solubility of water in SCFs is low (e.g., 0.31% v/v in $scCO_2$ at 50°C and 345 bar). If the water content increases during an enzymatic synthetic reaction (e.g., esterification), dehydrating agents (e.g., molecular sieves) may be used to prevent decay in the synthetic activity. The actual amount of water needed is specific to each solvent–substrate–enzyme system and must be maintained at constant level throughout the process ***(3)***. In this context, the support used to immobilize the enzymes and the hydrophobicity of the SCF also need to be considered. As an example, Kamat et al. ***(10)*** pointed to the increased activity that could be obtained for the lipase-catalyzed alcoholysis of methyl methacrylate by increasing the hydrophobicity of the SCF in the same conditions (45°C and 110 bar): SF_6 > propane > ethane > ethylene > CHF_3 > CO_2. In the case of $scCO_2$, it has also been observed how its logP parameter changed from 0.9 to 2.0, which involved an increase in solvent hydrophobicity, as a consequence of increasing the pressure from 30 to 118 bar at 50°C ***(14)***. In all cases, the increase in SCF hydrophobicity produced a reduction in water solubility. However, one limitation of $scCO_2$ is the fact that it preferentially dissolves hydrophobic compounds, although strategies—involving complexation with phenylboronic acid ***(9)*** or the addition of surfactants ***(4)***—can be developed to dissolve hydrophilic materials in this SCF.

Furthermore, the poor stability exhibited by free or immobilized enzymes in SCFs is probably the main drawback of these solvents in industrial biocatalytic processes ***(4)***. In the case of $scCO_2$, several adverse effects on enzyme activity and stability have been described ***(13)***. These effects have been attributed to local changes in the pH of the hydration layer ***(8,10)***, or by conformational changes produced during the pressurization/depressurization steps ***(12,15)***, as well as by the ability of CO_2 to form carbamates with free amine groups on the protein surface, resulting in changes in the secondary structure ***(11,16)***. Recently, a new procedure for immobilizing enzymes by sol-gel entrapment in silica-aerogels has been described, whereby enzyme molecules are included within a rigid glass framework, and this has a clearly stabilizing effect against enzyme deactivation by $scCO_2$ ***(17)***. However, the best results have been obtained using ionic liquids (ILs; *see* Chapter 23). The excellent ability of these neoteric solvents to overstabilize enzymes (e.g., free CALB, Novozymes®, among others) has also been observed for an $scCO_2$ reaction medium, even under extremely harsh conditions (e.g., 150°C and 100 bar) ***(18–21)***. ILs have been shown to act as suitable media and liquid-supports for immobilizing enzymes molecules, providing an adequate microenvironment for many enzyme-catalyzed chemical transformations in nonaqueous conditions. Additionally, the exceptional ability of $scCO_2$ to extract a wide variety of hydrophobic compounds from certain ILs has been clearly demonstrated because, although $scCO_2$ is highly soluble in the IL phase, the same IL is not very soluble in the $scCO_2$ phase ***(22)***. Thus, biphasic biocatalytic processes (e.g., ester synthesis, the kinetic resolution of *sec*-alcohols) in IL/$scCO_2$ systems have provided excellent results, where a homogeneous enzyme solution is immobilized in the liquid phase, and substrate and products reside largely in the supercritical phase ***(18–24; see* Fig. 2**). This strategy, whereby the mass-transfer between both IL and $scCO_2$ phases need to be optimized ***(23)***, constitutes an interesting way for designing integral green synthetic chemical bioprocess that provide pure products.

Several types of enzymatic reactor, including stirred-tank ***(8–15,17)***, continuous packed bed ***(6,18–21,23,24)***, or membrane ***(25–27)*** reactors have been applied in SCFs, where they can be used with either free or immobilized enzymes, with and without ILs. The design of SCF bioreactors is another key feature, where mass-transfer limitations, environmental conditions (pressure and temperature), and products can easily be recovered. As example, Marty et al. ***(6)*** developed a recycling packed bed enzyme-reactor at pilot scale for Lipozyme®-catalyzed ethyloleate synthesis by esterification from oleic acid and ethanol in $scCO_2$. The proposed system was coupled with a series of four high-pressure separator vessels, in which a pressure cascade was brought about by back-pressure valves, allowing continuous recovery of the liquid product from the bottom of each separator and then the recycling of unreacted substrates. Furthermore, membrane reactors represent an attempt to integrate catalytic conversion, product separation and/or concentration, and catalyst recovery in a single operation. Thus, Lozano et al. ***(27)*** reported how enzymatic dynamic membranes, formed by depositing water-soluble polymers (e.g., gelatin or polyethyleneimine) on a ceramic porous support, exhibited excellent properties for continuous synthetic processes in $scCO_2$, including a high degree of operational stability which favored reuse.

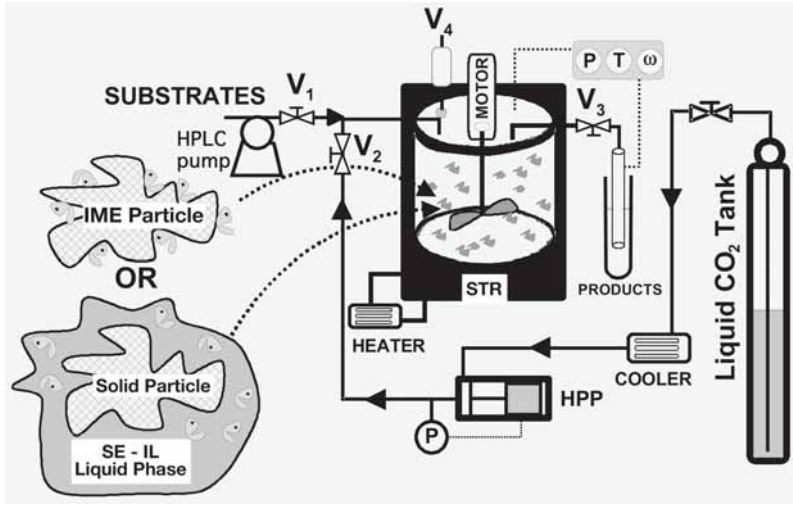

Fig. 2. High-pressure stirred tank reactor for enzyme-catalyzed transformations in $scCO_2$. V_1 to V_3, needle valves; V_4, safety valve; HPP, high pressure pump.

2. Materials

2.1. High-Pressure Stirred-Tank Reactor (see Fig. 2)

1. Liquid carbon dioxide tank (grade 4, 99.99 % minimum purity, water content lower than 10 ppm; *see* **Notes 1–3**).
2. Cooler.
3. High-pressure pump (HPP; Dosapro-Milton-Roy model Milroyal B).
4. Heater.
5. High-pressure stirred-tank reactor (Paar [www.paarinst.com], model 4560, 300 mL overall volume) equipped with needle valves and pressure, temperature, and stir rate controls.
6. High-performance liquid chromatography (HPLC) pump (Shimadzu Biotech, model LC-10AT, www.shimadzu.com).
7. Thermostated restrictor, calibrated at 1.5 mL/min overall flow (Teledyne Isco, Lincoln, NB; *see* **Note 4**).

2.2. High-Pressure Packed-Bed Reactor (see Fig. 3)

1. Liquid carbon dioxide tank (grade 4, 99.99 % minimum purity, water content lower than 10 ppm) pressurized at 100 bar (*see* **Notes 1–3** and **5**).
2. Supercritical fluid extractor (Teledyne Isco, model SFX 220), equipped with a syringe pump (model 100DX, 100 mL overall volume), needle valves, and devices for controlling pressure, temperature, and flow rate.
3. HPLC pump (Shimadzu, model LC-10AT).
4. Thermostated restrictor, calibrated at 1.5 mL/min overall flow (Teledyne Isco) (*see* **Note 4**).

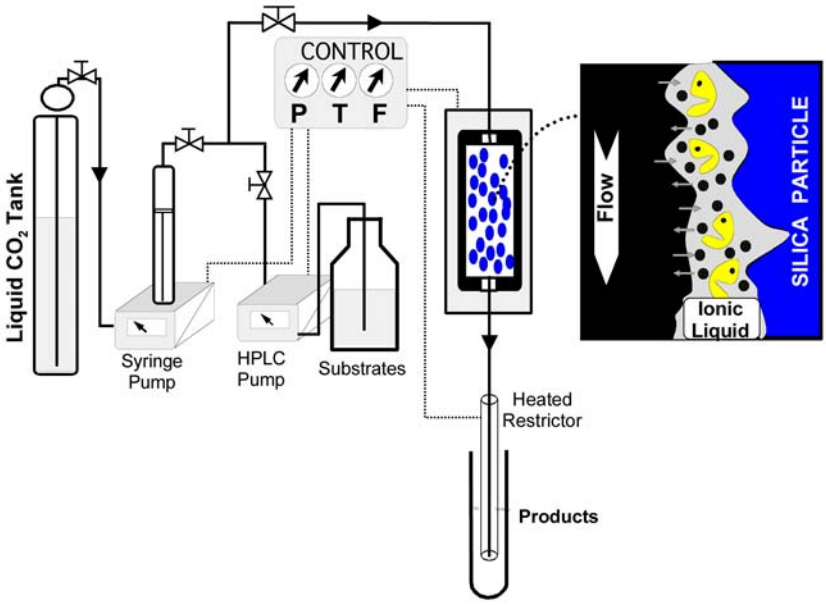

Fig. 3. Packed-bed reactor for continuous enzyme-catalyzed transformations in $scCO_2$, including the scheme of the biphasic nature of biotransformations in enzyme-IL-$scCO_2$ systems.

2.3. Cross-Flow Membrane Reactor for Enzyme Immobilization (see Fig. 4)

1. α-Alumina microporous tubular membrane (130-mm length, 7-mm i.d., 0.2-µm pore size, 28.6-cm^2 effective surface; Exekia, Bazet, France, www.exekia.com).
2. Aqueous solution of gelatin (5 mg/mL) containing 5 mg/mL polyethyleneimine (2 L).
3. Cross-flow filtration system for tubular ceramic membranes equipped with high-flow membrane pump (Hydra-Cell, model G037X, Wanner Engineering, Minneapolis, MN), needle valves and pressure gages (*see* **Note 6**).
4. 2% Glutaraldehyde solution (w/v) in 50 mL of 0.1 *M* carbonate buffer, pH 9.2.
5. 1 L of 0.1*M* phosphate buffer, pH 7.8.
6. *Candida antarctica* lipase B (CALB, cat. no. 525L, Novozymes, Krogshoejvej, Denmark) solution (15 mg protein/mL) 0.1 *M* phosphate buffer, pH 7.8.

2.4. High-Pressure Membrane Reactor With Recirculation (see Fig. 5)

1. Liquid carbon dioxide tank (grade 4, 99.99 % minimum purity, water content lower than 10 ppm; *see* **Notes 1–3**).
2. Cooler.
3. High-pressure pump (Model Milroyal B, Dosapro, Milton-Roy).
4. Heater.

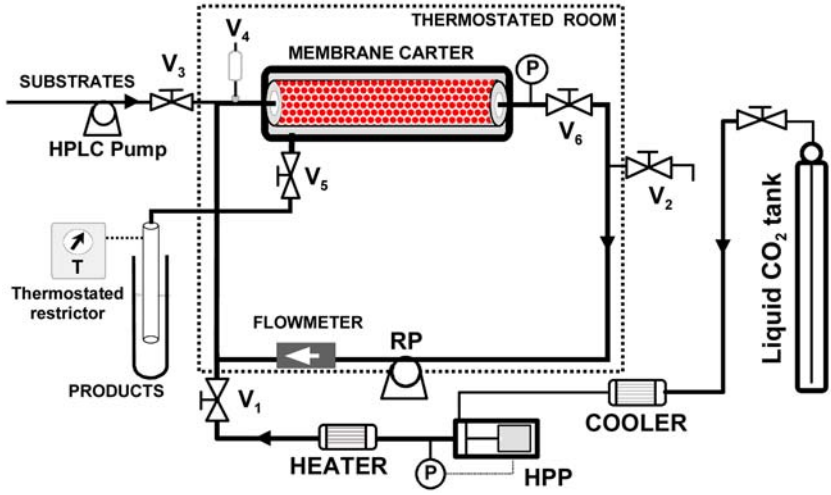

Fig. 4. High-pressure membrane reactor with recirculation for enzyme-catalyzed transformations in $scCO_2$. V_1, V_2, V_3, V_5 and V_6, needle valves; V_4, safety valve; HPP, High pressure pump; RP, recirculation pump.

5. High-pressure recirculation membrane reactor, constructed in stainless-steel (internal volume 125 mL), equipped with needle valves, pressure and flow controls, recirculation pump (model 219 Micropump) and mass-flowmeter (Rheonik, Munich, Germany, www.rheonik.com) and placed in thermostated room (*see* **Note 7**).
6. HPLC pump (model LC-6A, Shimadzu).
7. Thermostated restrictor, calibrated at 1.5 mL/min overall flow rate (Teledyne Isco; *see* **Note 4**).
8. Ceramic membrane containing immobilized CALB.

3. Methods

3.1. Novozymes®-Catalyzed Butyl Butyrate Synthesis in $ScCO_2$ Using a Stirred-Tank Reactor (27)

1. Switch on the cooler (0°C), the heater (40°C) and the thermostated restrictor (50°C) of the system for 30 min in advance.
2. Place 50 mg Novozymes 435 (a commercial immobilized CALB preparation) in the reactor tank and carefully close the system (*see* **Note 8**).
3. Open valve V_2 and closed valves V_1 and V_3, and then open the CO_2 tank to fill the reactor to the same pressure as the bottle (*see* **Fig. 2**).
4. Switch on HPP to fill the reactor with pressurized CO_2 until reach a pressure of 100 bar is reached in the reactor. Then, close valve V_2 and quickly stop the HPP (*see* **Notes 9–11**).
5. Switch on the mechanic stirrer at 150 rpm.

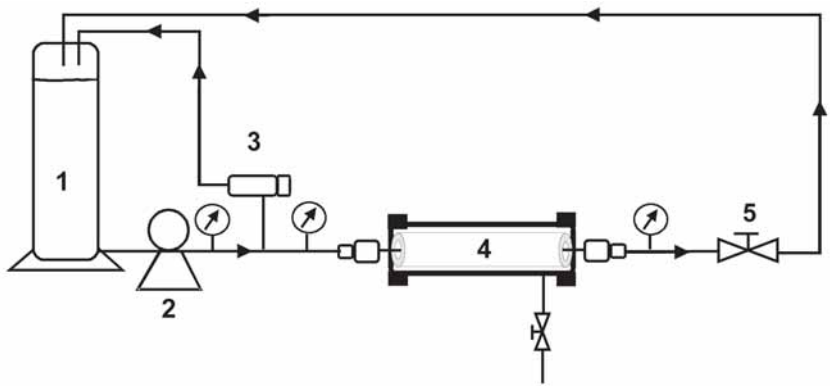

Fig. 5. Cross-flow filtration system for tubular ceramic membranes. (1) feed tank, (2) membrane pump, (3) safety valve, (4) membrane carter, (5) needle valve.

6. To start the reaction, open valve V_1 and introduce 2 mL of an equimolar solution of pure substrates (4.54 *M* vinyl butyrate and 1-butanol, respectively) at 2 mL/min flow rate by using the HPLC pump (for exactly 1 min). Then, stop the pump and immediately close the valve V_1 (*see* Chapter 23).
7. To follow the reaction, aliquots could be taken from the reaction mixture at appropriate intervals of time. Carefully open valve V_3 and bubble the reaction mixture into a controlled amount of hexane (e.g., 2 mL) placed on an ice-bath for exactly 3 min. Then, close the valve V_3. Full reaction occurs at 3 h, giving a 99.5% synthetic product yield (*see* **Note 12**).
8. If necessary, pump additional CO_2 into the reactor to reach and/or maintain desired pressure (e.g., 100 bar; *see* **Note 8**).
9. Mix 500 µL of hexane extract with 500 µL of 10 m*M* propyl acetate (internal standard) solution in hexane, and chromatographically analyzes 1 µL of the resulting solution.
10. Analysis of samples can be carried out using a gass chromatography (GC) semicapillary Nukol column (15 m × 0.53 mm × 0.5 µm; Supelco, Bellefonte, PA, www.sigmaaldrich.com) and a flame ionization detector. Chromatographic conditions are as follows: carrier gas (N_2) at 8 kPa (20 mL/min total flow); temperature programme: 45°C, 4 min, 8°C/min, 133°C; split ratio, 5:1; detector, 220°C. Retention times of peaks are as follows: propyl acetate, 3.2 min; vinyl butyrate, 4.3 min; 1-butanol, 6.6 min; butyl butyrate, 7.7 min and butyric acid, 13.5 min.

3.2. Free CALB-Emim NTf_2-Catalyzed Kinetic Resolution of rac-1-Phenylethanol in $ScCO_2$ Using a Continuous Packed-Bed Reactor (17)

1. Into a test tube containing 2 mL of $emim^+$ NTf_2^-, add 65 µL of 0.9% (w/v) CALB solution (cat. no. 525L, Novozymes) and shake the mixture mechanically at room temperature until a clear solution is obtained (*see* Chapter 23).

2. Fill a Teledyne Isco 220 SFX cartridge (10-mL total volume) with glass-wool (2 g). Then, add the enzyme-emim$^+$ NTf_2^- solution to wet the glass-wool, and finally place the cartridge in the high pressure extraction apparatus.
3. Open CO_2 tank to fill the syringe pump at the desired pressure (e.g., 100 bar) (*see* **Fig. 3**).
4. Program the Teledyne Isco system as a dynamic extraction process at constant pressure (100 bar) and temperature (40°C).
5. Simultaneously switch on both the Teledyne Isco system and the HPLC pump to start the process by introduction of an equimolar solution of pure substrates (4.28 *M* vinyl propionate and *rac*-1-phenylethanol, respectively) at 0.01 mL/min flow rate (42.8 μmol/min mass-flow for each substrate; *see* **Fig. 7** for reaction mechanism).
6. The system automatically opens the exit valve, continuously bubbling the reaction mixture through a calibrated heated restrictor (1 mL/min, 60°C) in a controlled amount of hexane (e.g., 2 mL) previously placed on an ice-bath. To follow the reaction kinetic, substitute the hexane every 30 min for analysis (*see* **Note 13**).
7. Mix 500 μL of hexane extract with 50 μL of 100 m*M* butyl butyrate (internal standard) solution in hexane, and chromatographically analyze 1 μL of the resulting solution.
8. Analysis of samples can be carried out by using a GC beta-DEX-120 capillary column (30 m × 0.25 mm × 0.25 μm, Supelco) and a flame ionization detector. Chromatographic conditions are as follows: carrier gas (He) at 1 MPa (205 mL/min total flow); temperature program: 60°C, 10°C/min, 130°C; split ratio, 100:1; detector, 300°C. Retention times of peaks are as follows: vinyl propionate, 3.2 min; propionic acid, 6.5 min; butyl butyrate, 7.3 min; *R*-1-phenylethanol, 15.4 min; *S*-1-phenylethanol, 16.0 min; *R*-1-phenylethyl propionate, 19.3 min.

*3.3. Immobilization of CALB on Ceramic Tubular Membranes (***see** ***Fig. 6 and ref. 25****)*

1. Place the α-alumina membrane in the carter (*see* **Fig. 4**), and then pump pure water at 20 L/min flow rate and room temperature to hydrate and wash the membrane.
2. Slightly close the recirculation needle valve to reach a backpressure of 2 bar in the system, allowing hydration of the pores.
3. Place the gelatin/polyethyleneimine solution in the feed tank and pump at 20 L/min flow rate and room temperature. The polymer film at the inner surface of the membrane is formed by cross-flow filtration of this solution at 2 bar backpressure for 60 min. The resulting filtrate solution is re-added to the feed tank during the process (*see* **Fig. 6**).
4. Open the system and carefully remove the ceramic membrane.
5. Fill the ceramic membrane with the glutaraldehyde solution and allow to react for 30 min at room temperature to activate the free amino groups.
6. Wash the membrane with water to remove the excess of glutaraldehyde.
7. Fill the activated ceramic membrane with 15 mg/mL CALB solution (Novozymes 525L) in 0.1 *M* phosphate buffer, pH 7.8, and keep overnight at 8°C.

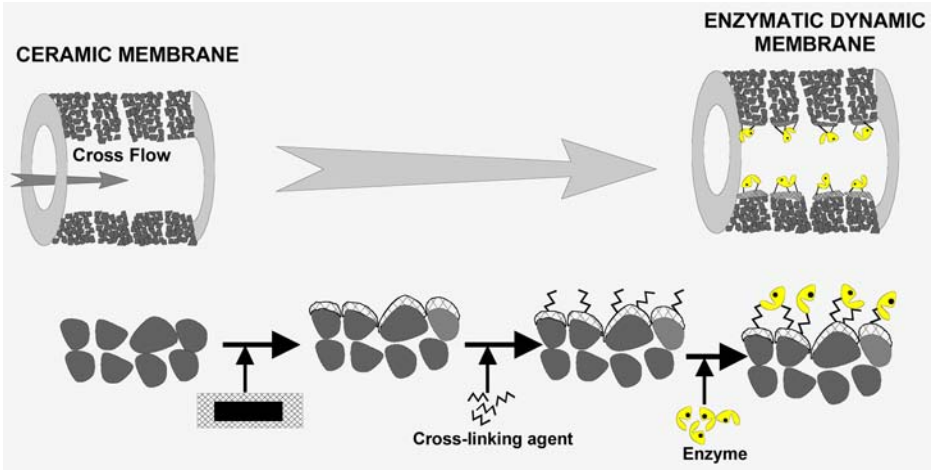

Fig. 6. Schematic procedure for the preparation of dynamic membranes with immobilized enzymes.

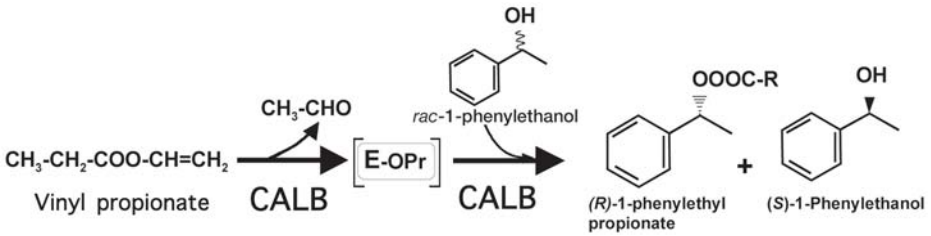

Fig. 7. Lipase-catalyzed kinetic resolution of *rac*-1-phenylethanol.

8. Collect the remaining enzyme solution and wash the enzymatic membrane twice with 0.1 *M* phosphate buffer, pH 7.8. All removed enzyme fractions can be used to quantify the immobilization yield ***(25)***.
9. Store the enzymatic membrane under dry conditions over P_2O_5 in a dessicator at 8°C.

3.4. Immobilized CALB-Catalyzed Butyl Butyrate Synthesis in $ScCO_2$ Using a Membrane Reactor With Recirculation (27)

1. Switch on the cooler (0°C), the heater (40°C), room thermostat (40°C), and the thermostatic restrictor (50°C) of the system for 30 min in advance.
2. Place the enzymatic ceramic membrane in the carter (*see* **Fig. 5**) and carefully close the system.
3. Open valves V_1 and V_5 and close valves V_2, V_3 and V_4, and then open the CO_2 tank to fill the circuit to reach the pressure of the bottle (*see* **Fig. 4**).

4. Switch on HPP to fill the reactor with pressurized CO_2 to reach a pressure of 90 bar in the reactor. Then, close valve V_1 and quickly stop the HPP (*see* **Notes 9–11**).
5. Switch on the recirculation pump (RP) and flowmeter to control mass-flow.
6. To start the reaction, open valve V_3 and introduce 2 mL of an equimolar solution of pure substrates (4.54 *M* vinyl butyrate and 1-butanol, respectively) at 2 mL/min flow rate using the HPLC pump (for exactly 1 min). Then, stop the pump and immediately close valve V_3 (*see* Chapter 23).
7. To follow the reaction, aliquots could be taken from the reaction mixture at appropriate intervals of time. Carefully open valve V_5 and bubble the reaction mixture into a controlled amount of hexane (e.g., 2 mL) placed on an ice-bath, for exactly 3 min. Then, close the valve V_5. Full reaction occurs at 4 h, giving a 99.5% synthetic product yield (*see* **Note 14**).
8. If necessary, pump additional CO_2 into the reactor to reach and/or maintain the desired pressure (e.g., 100 bar) (*see* **Note 8**).
9. Mix 500 µL of hexane extract with 500 µL of 10 m*M* propyl acetate (internal standard) solution in hexane, and chromatographically analyze 1 µL of the resulting solution.
10. The samples can be analyzed by a GC semicapillary Nukol column (15 m × 0.53 mm × 0.5 µm, Supelco) and a flame ionization detector. Chromatographic conditions are as follows: carrier gas (N_2) at 8 kPa (20 mL/min total flow); temperature program: 45°C, 4 min, 8°C/min, 133°C; split ratio, 5:1; detector, 220°C. Retention times of peaks are as follows: propyl acetate, 3.2 min; vinyl butyrate, 4.3 min; 1-butanol, 6.6 min; butyl butyrate, 7.7 min and butyric acid, 13.5 min.

4. Notes

1. Purity of carbon dioxide should be as least grade 4 (99.99 %). Optionally, CO_2 can be dried by placing in-line a special drying filter.
2. Carbon dioxide is an asphyxiating gas and the tank must be placed in a well-ventilated room
3. Carbon dioxide line should be is 1/4–stainless-steel tube.
4. A heater restrictor is necessary to prevent the exit-tube from freezing as samples are taken. For this, a 1/16 stainless-steel HPLC tube (1.5-m length) placed in a thermostatic bath can be used. A back-pressure regulator or two-series of needle valves must be placed to regulate the overall flow at the exit.
5. The CO_2 tank pressure is 55 bar. Hyper-pressurized CO_2 is obtained by capping the tank with helium until the desired pressure is reached; it is necessary to use a tank equipped with a siphon system to take CO_2 from the bottom of the bottle. The use of this kind of CO_2 tank is encouraged when high-pressure syringe pumps are used to reach supercritical conditions.
6. The cross-flow system will be constructed in 1/2–stainless-steel tube to support up 30 L/min recirculation flow without any backpressure in the system.
7. A safety valve must be included in the system, which is previously calibrated to the desired pressure (e.g., 100 bar).
8. Carefully close the reactor to avoid any loss of chemicals and/or CO_2 when the system is pressurized.

9. The HPP can only pump liquids (e.g., CO_2 at 0°C and 55 bar), but not gases.
10. To avoid a fall in the pressure close valve V_2 before stopping the HPP pump.
11. If pressure exceeds desired limit, the safety valve V_4 opens automatically to release the excess of CO_2.
12. To avoid loss of immobilized enzyme and prevent the needle valves from becoming blocked by particulate solids, a frit stainless-steel filter must be placed at the beginning of tube for taking samples (inside the reactor).
13. The system reaches a stationary state for synthetic product at 120 min, giving a 60% enantioselective product yield.
14. Alternatively, the thermostated restrictor can be placed in valve V_2 to take samples directly from the circuit.

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25

Immobilized Enzymes for Biomedical Applications

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Summary

Immobilized enzymes were first applied in the biomedical field aiming to detect bioactive substances or to treat a disease condition. This chapter presents two approaches used for the immobilization of L-asparaginase intended for the treatment of leukemia, based on the entrapment of this enzyme in a matrix. The particulate drug carriers described in this chapter are liposomes and nanoparticles, even though the methods described here could be used for the immobilization of other enzymes with therapeutic uses.

Key Words: Immobilization; L-asparaginase; liposomes; nanoparticles.

1. Introduction

The study of immobilized enzymes for biomedical applications started in the 1960s ***(1)***, aiming to solve some of the limitations to the use of enzymes in clinics, to make them more stable, less immunogenic and toxicologic, and to present a longer in vivo circulation lifetime ***(2)***. Since then, several approaches have been used in enzyme therapy either for the detection of bioactive substances in the diagnosis of diseases or with the aim to treat a disease condition, such as the correction of inborn metabolic defects, cardiovascular diseases, cancer, intestinal diseases, or for the treatment of intoxication (**Table 1**).

For the immobilization of enzymes two different approaches have been used ***(26)***, the first consisting in the cross-linking or the covalent attachment of the enzyme to a support (immobilization by binding) and the second based on the entrapment of the enzyme in a matrix (immobilization by inclusion).

Among the methods used for immobilization by binding, conjugation with polymers has received great attention in the last several years. A large number of polymers can be considered for enzyme immobilization, although the strict requirements needed for a biomedical approach limit this number significantly. The polymers used for enzyme immobilization may be fully biocompatible, and

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Table 1
Enzymes That Have Been Immobilized for a Clinical Purpose

Enzyme	Disease treated	Ref.
Alcohol dehydrogenase and acetaldehyde dehydrogenase	Alcohol intoxication	3,4
Asparaginase	Acute lymphocytic leukemia	5–8
Adenosine deaminase	Human melanoma and hepatocarcinoma	9,10
Cytochrome P450 (cells producing the enzyme)	Cancer therapy, to convert ifosfamide to its cytotoxic metabolite	11
β-Glucosidase	Gaucher's disease	12,13
β-Glucuronidase	Cancer therapy, for the activation of anticancer prodrugs	14
Glucose oxidase–peroxidase	Oral infections	15
Prolidase	Prolidase deficiency	16
t-Plasminogen activator	Myocardial infarction	17
Pepsine, chymotrypsin, trypsin	Replacement therapy in gastrointestinal diseases, treatment of fat malabsorption	18,19
Phosphotriesterase	Intoxication by organophosphates	20
Phenylalanine ammonia lyase	Phenylketonuria	21,22
Streptokinase	Thrombolytic therapy	23
Urease (*E. coli* cells engineered to produce urease)	Removal of urea in kidney failure	24,25

biodegradable, and should possess high purity and homogeneous molecular-weight (MW) distribution (2). Besides, one should bear in mind that covalent coupling of enzymes to polymers may result in conformational alterations and decrease significantly enzymatic activity (27).

One of the most popular polymers currently used for the modification of peptides and proteins with therapeutic potential is polyethylene glycol (PEG). PEG is inexpensive, biocompatible, nontoxic, and is already approved by drug regulatory agencies (28). Pegylation may be used to improve the properties of the enzymes without causing any loss in the activity of the enzymes, which could be later used as protein drugs or as catalysts in bioreactors (29). Currently, several enzyme/PEG conjugates are used in clinics; some of these are listed by Michel Vellard in a recent review (30) and comprise recombinant human tissue-plasminogen activator, adenosine deaminase, arginine deaminase, and asparaginase.

The immobilization by inclusion methods present some advantages over the methods used for the immobilization by binding: there is no need for derivatization of the enzyme (26), the systems provide a higher degree of protection against enzymatic degradation and other destructive factors, allow for much higher drug loads, and enzymes can be immobilized into the system in a more stabilized form, such as multienzymatic systems, enzyme mixtures, or even cells producing a certain enzyme (1).

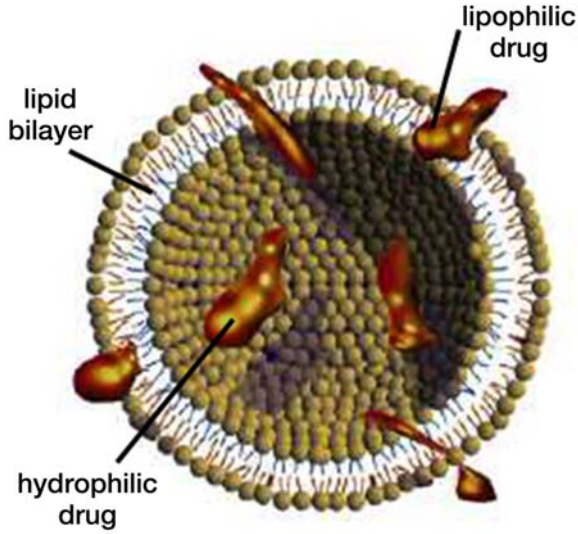

Fig. 1. Structure of a liposome. Hydrophilic drugs are encapsulated in the inner aqueous space, whereas lipophilic drugs are entrapped in the phospholipid membrane.

However, inclusion methods present disadvantages related to the use of synthetic materials, which can be solved by the use of biodegradable polymers (e.g., polymers and copolymers derived from lactic and glycolic acid, alginate, chitosan) or by the use of more biocompatible immobilization carriers such as liposomes or red blood cells ***(1,2)***.

Among particulate drug carriers, liposomes are the most extensively studied and possess the most suitable characteristics for polypeptide encapsulation. Liposomes are microscopic vesicles composed of membrane-like phospholipid bilayers enclosing aqueous compartments (*see* **Fig. 1**). They are biologically inert, biocompatible, and cause very little toxic or antigenic reaction. By the encapsulation of enzymes into liposomes, the in vivo blood circulation of enzymes can be increased and, moreover, the liposomes can mask their antigen determinants, avoiding the adverse immunological reactions ***(31)***.

An alternative to liposomes are controlled-release systems made of biodegradable polymers, such as nanoparticles. Micro/nanoparticles are polymeric colloidal systems, ranging in size from 10 nm to micrometers, in which the drug is dissolved, entrapped, encapsulated, or adsorbed (*see* **Fig. 2**). Nanoparticles, because of their versatility for formulation, sustained release properties, subcellular size, and biocompatibility with tissue and cells appear to be a promising system for the immobilization of enzymes and protein drugs ***(32)***.

One of the enzymes that has been immobilized in different supports and has received great attention from researchers is asparaginase. L-Asparaginase (EC 3.5.1.1) catalyzes the hydrolysis of L-asparagine (an essential amino acid) to L-aspartic acid and ammonia ***(33)***. Leukemic cells lacking asparagine synthase are unable to

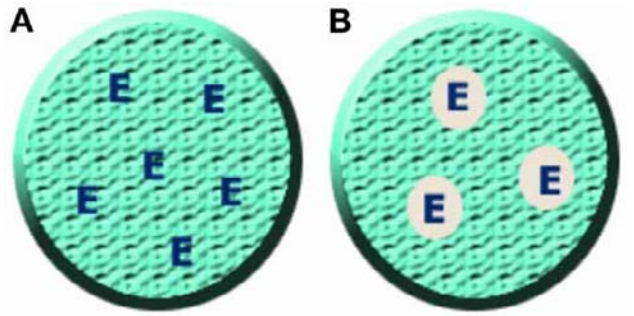

Fig. 2. Enzyme immobilization into polymeric micro- or nanoparticles (E enzyme). The entrapped enzyme may be dispersed **(A)** or dissolved **(B)** in the micro- or nanoparticulate system.

synthesize asparagine and are dependent on an exogenous source of asparagine for their survival. Treatment with L-asparaginase causes a rapid depletion of extracellular asparagines, leading to selective killing of the leukemic cells. Normal cells, able to synthesize asparagine intracellularly, are less sensitive to L-asparaginase oncolytic activity. L-Asparaginase therapy reduces the plasma levels of the amino acid and thus interferes in protein synthesis ***(34)***. **Figure 3** shows the mechanism of action of L-asparaginase.

L-asparaginase (usually from *Escherichia coli* or *Erwinia crysanthemi*) is often used in protocols for the treatment of leukemia in combination with vincristine, daunomycin, methotrexate, or cyclophosphamide ***(34)***. Morever, recent clinical trials have proven the efficacy of asparaginase for the treatment of other childhood lymphoid malignancies (i.e., lymphoblastic lymphoma) ***(35)***.

Although a wide experience has been obtained from the treatment of acute lymphoblastic leukemia (ALL) in recent years, still there are some limitations to the administration of L-asparaginase as a result of its short in vivo half-life, which implies the need for multiple injections in order to reach therapeutic levels of the enzyme, and to the immunological side effects, ranging in severity from mild allergic reactions to anaphylactic shock because this enzyme is a protein foreign to humans, especially upon the repetitive administration needed to reach therapeutic levels of the enzyme.

Therefore, alternative therapy or substitutes for L-asparaginase has been investigated over the past years. One of the approaches studied in clinics that has given successful results, with less immunogenicity and longer half-life, is binding to polyethylene glycol, originating a new molecule called pegaspargase. This approach is already approved by the FDA under the trade name of Oncaspar®. This modification leads to a less frequent administration and results in less immunogenic reaction ***(33)***. Nevertheless, some authors do not recommend routine substitution of L-asparaginase for pegaspargase owing to a possible higher incidence of pancreatitis associated with pegaspargase ***(36)***.

In this chapter, we will focus on the immobilization of L-asparaginase by inclusion into two delivery systems, namely liposomes and nanoparticles. These methods can be adapted for the immobilization of other therapeutic enzymes.

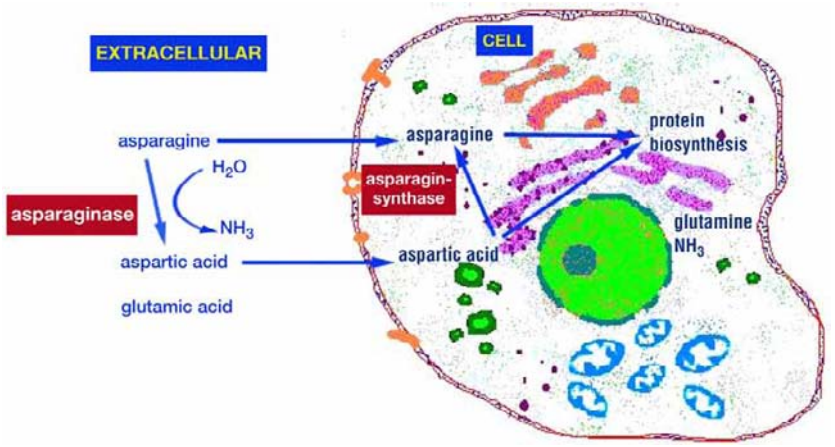

Fig. 3. Mechanism of action of L-asparaginase.

2. Materials

2.1. Immobilization of L-Asparaginase Into Polylactide-Coglycolide Nanospheres

2.1.1. Preparation of L-Asparaginase-Loaded Nanospheres

1. L-Asparaginase (from *E. coli*, Sigma, St. Louis, MO).
2. Polylactic-coglycolic acid 50:50 (PLGA; Resomer RG 503H, MW 34000 Da; from Boehringer Ingelheim, Ingelheim, Germany; *see* **Note 1**).
3. Ethyl acetate (Panreac Química, Barcelona, Spain)
4. Polyvinyl alcohol (PVA; MW 30,000–70,000, Sigma).
5. Phosphate buffer, pH 7.4.

2.1.2. Characterization of L-Asparaginase-Loaded Nanospheres

1. Sodium hydroxide (NaOH) (Panreac Química).
2. Sodium dodecyl sulphate (SDS; Sigma).
3. 66 m*M* Phosphate buffer, pH 7.4.
4. Pluronic F68 (BASF, Ludwigshafen, Germany).
5. Anhydrous glycerol (Sigma).
6. 50 m*M* Tris buffer, pH 8.45.
7 α-Ketoglutarate-sodium, L-glutamic oxaloacetate transaminase, L-malic dehydrogenase, NADH, and L-asparagine (Sigma).

2.2. Immobilization of L-Asparaginase Into Liposomes

2.2.1. Preparation of L-Asparaginase-Loaded Liposomes

1. Egg phospatidylcholine, cholesterol, and stearylamine (Sigma).
2. L-Asparaginase (Sigma).
3. Mannitol (Sigma).
4. NaCl (Panreac Quimica).

2.2.2. Characterization of L-Asparaginase-Loaded Liposomes

1. Triton X-100 (Sigma).
2. SDS (Sigma).
3. 66 m*M* Phosphate buffer, pH 7.4.
4. Pluronic F68 (BASF).
5. Anhydrous glycerol (Sigma).
6. 50 m*M* Tris buffer, pH 8.45 with 0.1 *M* HCl.
7. α-Ketoglutarate-sodium, L-glutamic oxaloacetate transaminase, L-malic dehydrogenase, NADH, and L-asparagine (Sigma).

3. Methods

3.1. Immobilization of L-Asparaginase Into Polylactide-Coglycolide Nanospheres

3.1.1. Preparation of L-Asparaginase-Loaded Nanospheres

L-Asparaginase loaded nanospheres can be prepared by the water-in-oil-in water solvent evaporation technique ***(5)***.

1. Transfer 0.1 mL of 5% L-asparaginase aqueous solution to 0.5 mL ethyl acetate containing 50 mg PLGA placed a 10-mL glass tube (*see* **Note 2**).
2. Emulsify the mixture by sonication for 15 s (Branson Sonifier 250 at output 2 [15 W]; *see* **Note 3**).
3. Emulsify again by sonication the resulting emulsion (w/o) (same conditions as in **step 2**) in 2 mL of 1% PVA under cooling in an ice bath to form the second emulsion ($w_1/o/w_2$).
4. Dilute this double emulsion into 50 mL of 0.3% PVA and maintain the system under magnetic stirring for 15 min.
5. Transfer the suspension to a 50-mL round bottom flask and evaporate the solvent in a rotary evaporator (Rotavapor Buchi B-480) in order to accelerate the evaporation of the organic solvent (ethyl acetate).
6. Separate the nanoparticles with ultracentrifugation at 30,000*g* for 15 min (repeat the procedure three times in order to wash the nanoparticles).
7. Resuspend the nanoparticles in 20 m*M* phosphate buffer, pH 7.4.

3.1.2. Determination of Encapsulation Efficiency

The encapsulation efficiency (EE) indicates the percentage of the enzyme encapsulated with respect to the total amount used for the preparation of the nanoparticles.

1. Weight 20 mg of enzyme-loaded nanoparticles and shake them overnight in 6 mL of 0.1 *M* NaOH containing 2% sodium dodecyl sulfate (SDS). This allows for the polymer dilution and the release of the encapsulated enzyme.
2. Determine the amount of enzyme by the Lowry–Folin technique ***(37)***.

3.1.3. Determination of L-Asparaginase Activity

1. The biological activity of the enzyme is determined by the method described by Jayaram ***(38)***. The enzymatic activity of one unit L-asparaginase corresponds to formation of 1 mmol NAD/min at 37°C.

2. The enzyme-loaded nanoparticles are disintegrated by high-speed homogenization. Fifty-three milligrams of nanoparticles are suspended in 4 mL distilled water and homogenized with an Ultraturrax T-25 (IKA Labortechnik, Staufen, Germany) for 10 min in an ice bath.
3. The tip is rinsed with 1.5 mL distilled water and the homogenized suspension is extracted by end-over-end rotation for 1 h, with 4 mL 66 m*M* phosphate buffer (pH 7.4) containing Pluronic F68. The enzymatic activity of the supernatant is then assayed.
4. Prepare the reagent from 100 mL anhydrous glycerol, 50 mL 0.5 *M* Tris buffer, pH 8.45, 50 mg α-ketoglutarate-sodium, 180 U L-glutamic oxaloacetate transaminase, 110 U L-malic dehydrogenase, and 50 mg NADH adjusted to 500 mL with deionized water.
5. Immediately prior to use, 10 m*M* L-asparagine in 50 m*M* Tris-HCl buffer, pH 8.45, are added to the solution to a ratio 9:1 (v/v).
6. Incubate 2 mL reagent and 0.5 mL L-asparaginase-containing sample for exactly 1 h at 37°C.
7. Determine the decrease in absorption at 340 nm (Hewlett-Packard UV-VIS spectrophotometer, model HP8452A).

3.2. Immobilization of L-Asparaginase Into Liposomes

3.2.1. Preparation of L-Asparaginase-Loaded Liposomes

L-Asparaginase-loaded liposomes can be prepared by a dehydration–rehydration method (DRV) ***(6)***.

1. Weigh out the cholesterol into a 50-mL round bottom flask. Then add the phospholipids (phospatidylcholine and stearylamine) dissolved in 3 mL chloroform and allow the cholesterol to dissolve by gentle swirling. The lipid concentration is 18 μmol with a molar ratio of phospatidylcholine:cholesterol:stearylamine 7:2:0.25 (*see* **Note 4**).
2. Dry the lipid mixture in a rotary evaporator (Rotavapor Buchi B-480) under an N_2 stream to maintain an inert atmosphere.
3. Add slowly 1 mL of the L-asparaginase solution (0.03%) with gentle stirring in order to hydrate the thin film of lipid formed and form multilamellar vesicles. The stirring is continued for 2 h and should not be too vigorous in order to avoid heat generation (*see* **Note 5**).
4. The liposome suspension is lyophilized in a freeze-dryer (Cooling HetoTrap® HETO Model CT 110) overnight.
5. Following freeze-drying, the preparation is rehydrated with 0.1 mL of 0.3 *M* mannitol and the mixture is gently stirred in a vortex (*see* **Note 6**).
6. Leave the suspension at room temperature for 30 min.
7. The volume of the suspension is brought to 1 mL by adding 0.154 *M* NaCl.
8. Nonencapsulated enzyme is removed by three cycles of 30-fold dilution at 38,000*g* for 30 min.
9. Finally, the liposome pellet is resuspended in 1 mL of 0.154 *M* NaCl (*see* **Note 7**).

3.2.2. Determination of L-Asparaginase Recovery in Liposomes

Protein recovery is the liposome-associated protein in the final liposome suspension related to the total protein initially added, and indicates the yield of the process.

$$\text{Protein recovery (\%)} = \frac{\text{final liposome-associated protein } (P_f)}{\text{total initial protein } (P_i)} \times 100 \quad (1)$$

1. Two milliliters of liposome suspension are mixed with 2 mL of 2% (v/v) Triton X-100 and 0.5% SDS aqueous solution in order to disrupt the membranes and release the enzyme.
2. After that, the protein is determined using the method described by Lowry ***(37)***.

3.2.3. Determination of L-Asparaginase Encapsulation Efficiency

Encapsulation efficiency is the percentage of the ratio between the final protein-to-lipid (P/L) ratio and the initial P/L ratio.

$$\text{EE} = \frac{P_f / L_f \text{ in liposome suspension}}{\text{total } P_f / L_f \text{ in initial mixture}} \times 100 \quad (2)$$

1. Two milliliters of liposome suspension are mixed with 2 mL of 2% (v/v) Triton X-100 aqueous solution in order to disrupt the membranes.
2. After that, the protein is determined using the method described by Lowry ***(37)*** and lipid determinations are performed using the method described by Rouser et al. ***(39)***.

3.2.4. Determination of L-Asparaginase Activity

The biological activity of the enzyme is determined by the method described by Jayaram ***(38)*** after disruption of the liposomes with Triton X-100 as described in the previous section. The enzymatic activity of one unit L-asparaginase corresponds to formation of 1 mmol NAD/min at 37°C.

4. Notes

1. Although different types of the polymer PLGA may be used, a higher encapsulation efficiency of the enzyme is obtained with the PLGA Resomer 503H (with free carboxyl-end groups).
2. The organic solvent used to dissolve the polymer, ethyl acetate, has been reported to maintain L-asparaginase activity. Moreover, the loss of enzyme activity has been shown to be dependent on the initial concentration of the enzyme. At higher concentrations the activity is better preserved.
3. The sonication energy used in this step is gentle enough to preserve enzyme activity.
4. Different lipids and methods can be used for the preparation of enzyme-containing liposomes. However, the encapsulation efficiency, enzymatic activity, and protein-to-lipid ratio are found to be highly dependent on the lipid composition used.
   a. If dicetyl phosphate is used, a loss of enzymatic activity is observed.
   b. The addition of stearylamine increases the encapsulation efficiency up to 100%, with only a mild loss of enzymatic activity.
   c. Low-encapsulation efficiencies are observed with lipid mixtures of dimyristoylphosphatidylcholine (DMPC) with dioleoyl-phosphatidylcholine (DOPC).

5. The temperature of the aqueous phase must be higher than the main lamellar chain-melting phase transition temperature (T_m) of the phospholipid mixture.
6. The use of 0.3 *M* mannitol instead of deionized water in the rehydration step leads to an improved recovery, since it maintains a physiological intraliposomal osmolarity.
7. As an alternative of DRV liposomes, VET (extruded vesicles) liposomes can be made, although the encapsulation efficiency of the latter is much lower.

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26

Characterization of Immobilized Enzymes by Microcalorimetry

Ezio Battistel and Giovanni Rialdi

Summary

Direct information on the stability and biological activity of immobilized proteins can be obtained from calorimetry. This technique is flexible enough to give insight on the thermodynamic consequences of the immobilization in most experimental conditions, ranging from multipoint covalent attachment to simple absorption. Different calorimetric techniques can be tailored to study the different aspects of the protein chemistry, depending on the physical environment and the type of confinement. From differential scanning calorimetry experiments the thermodynamic parameters (the middle point temperature, the enthalpy change) of the unfolding transition of either the immobilized and free protein can be derived. Isothermal batch and flow calorimetry can assess the effects of the immobilization, support environment, and the type of entrapment on the active site reflected as differences in the binding capacity of specific ligands. Both techniques are suitable to determine reaction enthalpy changes and equilibrium constants from full protein–ligand titration curves. Selected examples of proteins immobilized by covalent single-point attachment in aqueous solutions and by absorption in organic solvents will be described and discussed in detail.

Key Words: Differential scanning calorimetry; flow and batch titration calorimetry; ligand binding; protein unfolding.

1. Introduction

Calorimetry is a useful tool to obtain direct information about the properties of immobilized proteins ***(1)***. Unlike other spectroscopic techniques, it has the advantage of being rather insensitive to the presence of the supports that are commonly used to immobilized proteins. Microcalorimetry can precisely address two fundamental questions about immobilized proteins: first, how does the stability of the protein change after immobilization? Second, how much the biological activity is affected? Let us consider these two questions separately.

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Concerning the first dilemma, calorimetric results can be related to a true thermodynamic definition of stability, not only for proteins but also for other biological complex systems (i.e., nucleic acids). If stability is defined as an equilibrium between the folded, native (N), and unfolded (U) forms of a protein,

$$N \quad U$$

then the thermodynamic parameters of the reaction (e.g., free energy, enthalpy and entropy changes) truly define the concept of stability. This definition is different from that used for kinetic experiments, where the biological activity is followed as a function of time in destabilizing conditions. In this case the reaction studied may be written as

$$N \quad U \rightarrow I$$

where I is the irreversibly inactivated form of the protein or enzyme. The time dependence of the activity gives information on the overall $N \rightarrow I$ transition. These studies are missing a fundamental detail of the inactivation process (i.e., the unfolding reaction $N \quad U$), although they may be most interesting to the biotechnologists because of their practical consequences. Moreover, the $N \rightarrow I$ transition often does not follow a simple single order kinetics, adding difficulties to the interpretation of the results. It can be argued that the unfolded state U may not be so well defined from the structural point of view as the N state. However, this is not a real problem insofar as the "unfolded state" (which can be only be a major "conformational change" of the molecule) is a well defined state from thermodynamic point of view. As a matter of fact, the reversible $N \quad U$ transition is well approximated by the two-states model ***(2)***.

Unfolding can be studied by differential scanning calorimetry (DSC). This technique gives directly the thermodynamic parameters of the unfolding transition, such as melting temperature, enthalpy, entropy and free energy change of the unfolding as well as the heat capacity changes. A comprehensive literature survey on protein thermodynamics, including the calorimetric results, has been collected by Pfeil ***(3)***.

How much is the biological activity affected by immobilization? Besides the catalytic activity of enzymes, biological reactions (such as ligand affinity or exchange, ion binding, and intermolecular interactions) can assess the integrity of the response of the biological macromolecule. In these cases, the most direct use of the calorimetric techniques is the determination of the heat change of the reaction (i.e., the reaction enthalpy change). This can be accomplished directly and precisely with a limited set of measurements, less than those currently required with other techniques (i.e., spectroscopic techniques) based on the study of the temperature dependence of the equilibrium constant of the reaction (van't Hoff methods). Isothermal batch calorimetry is the most suitable technique to determine enthalpy changes. Moreover, besides the enthalpy change, equilibrium data (i.e., the equilibrium constants) can be obtained without the use of other independent variables since the enthalpy change itself may be used as a variable reflecting the reaction progress. In these cases, titration calorimetry affords a full protein–ligand titration curve from a single experiment.

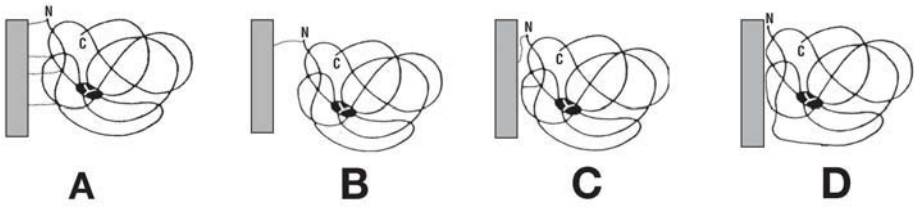

Fig. 1. Schematic representation of the protein macromolecule immobilized to the support by multipoint attachment (**A**), single-point attachment (**B**,**C**), and adsorption (**D**)

By comparison with the free enzyme in the same conditions, the direct assessment of the integrity of the protein-binding site can be obtained. Whatever the effects of the immobilization, support environment, or type of linking or entrapment on the active site, it will show up directly as a difference in the thermodynamic reaction coordinates. Thus equilibrium constants may be evaluated also in the presence of turbid, heterogeneous, multiphase, or multicomponent systems otherwise very difficult to study.

Protein immobilization can be accomplished mainly in three ways: covalent attachment to solid support, adsorption to surfaces, and entrapment in gels. The last will not be treated in this chapter. The first two methods are schematically shown in **Fig. 1**.

Enzymes can be covalently linked to a specifically functionalized surface by means of chemical bonds, whose number can vary from one (*see* **Fig. 1B**,**C**) through several (*see* **Fig. 1A**), or adsorbed on surfaces (*see* **Fig. 1D**). Single-point attachment may be obtained by carefully selecting the coupling conditions that depend on the reagents used.

In multipoint attachment (*see* **Fig. 1A**), the enzyme molecule is rigidly anchored to the support. The enhanced stability toward temperature increase and concentration of denaturants is often accompanied by a concurrent decrease of the catalytic or binding efficiency ***(4–6)***. This is a result of various factors such as an increased rigidity of the macromolecule, restricted accessibility of the binding or catalytic site, and uneven distribution of the attachment points.

Single-point attachment (*see* **Fig. 1B**) may give more "spatial freedom" to the anchored protein, without substantial limitations to the substrate accessibility with respect to the free molecule. However, this does not mean that interactions between macromolecule and the support surface (*see* **Fig. 1C**) do not occur. On the contrary, as we will see, this is by far the most common situation ***(7)***.

It is worth noting that interactions between protein and surface are unavoidable anyway, as clearly shown by visual inspection of **Fig. 2**. In this figure, the optimized simulated assembly of ribonuclease bound to CPC, controlled pore glass beads (glutaraldehyde C_5 spacer arm) is shown. Atomic distances were refined by allowing the spacer arm to freely rotate. As can be seen, the molecule is quite close to the support surface, which implies that a great deal of the time during the macromolecule positional fluctuations is spent in the proximity of the surface. In the protein molecule, it is possible to recognize two structural lobes (domains)

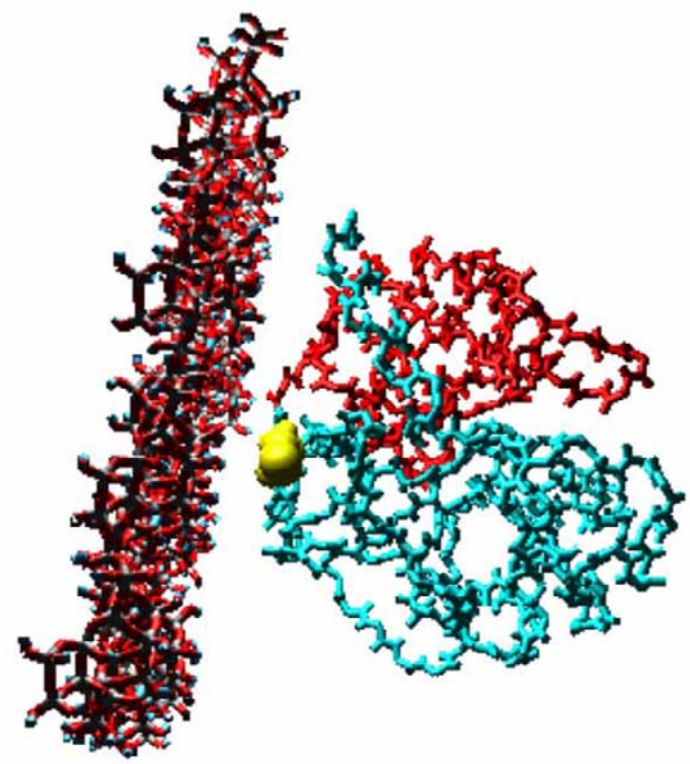

Fig. 2. RNase A molecule linked to controlled pore carrier (CPC, aminopropyl derivative) glass surface. The linking reagent is glutaraldehyde (C_5) coupled to the terminal lysine amino group. The assembly has been simulated with Insight II (Biosym, San Diego, CA).

divided by a deep cleft where the active site is located. As we will see, unlike in the native enzyme, these two domains will unfold as two independent units after immobilization.

A calorimetric approach to the study of immobilized proteins according to the different situations shown in **Fig. 1** will be presented in detail in the following sections. A notable exception will be the multipoint attachment case, which has already been presented in the literature ***(4)***. For clarity sake, ribonuclease A will be selected as a protein reference case for all the techniques. However, detailed thermodynamic and calorimetric analysis of the properties of several other unrelated immobilized proteins, such as papain ***(8)***, α-chymotrypsin ***(9)***, α-chymotrypsinogen ***(10)***, and lipase ***(11)*** can be found in the literature.

1.1. Single-Point Attachment: Thermal Unfolding

1.1.1. DSC Results

Sepharose and other natural gels (e.g., cellulose, κ-carrageenan, calcium alginate) are easily contaminated by bacterial growth during the manipulations. There-

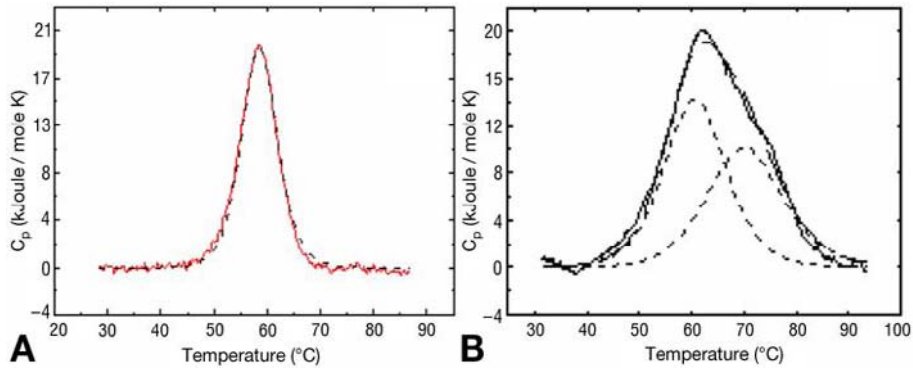

Fig. 3. Temperature dependence on the excess specific heat capacity of RNase. Free enzyme in 50 m*M* acetate buffer, pH 5.0 **(A)**; immobilized enzyme on CPC-silica through glutaraldehyde coupling, acetate 50 m*M*, pH 5.0 **(B)**. Continuous curves are the experimental recordings. Dotted curves are the deconvolution best fit.

fore, other synthetic polymers or reactive glasses (silica) were preferred. Ribonuclease A (RNase A) was immobilized on silica (glass) beads (CPC, controlled pore carrier, aminopropyl derivative) through glutaraldeyde-mediated chemical coupling. In **Fig. 3** the thermal unfolding transitions of the free (*see* **Fig. 3A**) and immobilized (*see* **Fig. 3B**) enzyme studied by DSC are shown. The transition parameters (the unfolding middle point temperature, T_m, and the enthalpy change, ΔH) obtained from the deconvolution of the thermogram for the free and immobilized enzyme, respectively, are listed in **Table 1**.

The unfolding transition of the free enzyme (*see* **Fig. 3A**) can be well approximated by the two-states model. This is confirmed by a value of the co-operativity unit, Cu (i.e., the ratio of the van't Hoff to the calorimetric enthalpy changes), very close to 1 (**Table 1**) ***(2)***. This does not apply to the overall transition of the immobilized enzyme. In this case, two independent overlapping transitions (each with a Cu close to 1) (**Table 1**) are necessary to fit the experimental curve ***(12)***. This result implies that, as a consequence of the immobilization, the protein cannot be considered a single thermodynamic domain but the sum of two independent parts (domains) having different stabilities. Note that one domain of the protein has a T_m of about 70°C, higher than the T_m of the native enzyme (59°C). Stabilization of part of the macromolecule is therefore achieved by interdomain dissociation. However, the integrity of the macromolecule is somehow preserved because the overall ΔH is similar, or even slightly higher (**Table 1**), to that of the free enzyme. The presence of a different local environment near the domain chemically linked to the support (e.g., **Fig. 1C**), may modify interdomain free energy contributions causing domains to uncouple ***(13)***.

The binding of the inhibitor 3'-cytidine monophosphate (3'-CMP) slightly stabilized the protein as suggested by a small increase of both T_m and ΔH.

Table 1
Enthalpy of Unfolding

	T_{m1} (°C)	ΔH_1 (kJ/mol)	Cu_1	T_{m2} (°C)	ΔH_2 (kJ/mol)	Cu_2	ΔH_{tot} (kJ/mol)
RNase free	58.9 ± 0.3	375 ± 6	0.99				375
RNase free + 3'-CMP	62.9 ± 0.3	439 ± 6	0.85				439
RNase CPC	60.0 ± 0.5	232 ± 8	0.99	70.1 ± 0.5	195 ± 7	1.02	427
RNase CPC + 3'-CMP	61.4 ± 0.4	261 ± 8	0.90	71.3 ± 0.5	200 ± 7	0.98	461

Note: ΔH, and middle-point transition temperature, T_m, of free and glutaraldehyde-immobilized RNase A at pH 5.0, acetate buffer 50 m*M*. The subscripts 1 and 2 refer to domain 1 and 2 in the immobilized protein. 3'-cytidine monophosphate (3'-CMP) is an enzyme inhibitor. (From **ref. *25***. Copyright 2005, American Chemical Society.)

1.2. Single-Point Attachment: Binding Studies

1.2.1. Isothermal Titration Calorimetry

The support (CPC silica glass beads) containing RNase was loaded into the calorimetric cell and titrated with a solution of 3'-cytidine monophosphate (3'-CMP). 3'-CMP is a RNase inhibitor, able to bind tightly to the protein active site. The titrating solution was continuously added with an external peristaltic pump, whereas the heterogeneous phase within the calorimetric cell was slowly stirred. The experimental heat evolved (W, watts) of the binding of 3'CMP to immobilized RNase as a function of time is presented in **Fig. 4**, curve *A*. The calorimetric output has been corrected for the instrumental time response delay (Tian effect, **Fig. 4**, curve *B*) ***(18)***. The thermodynamic parameters obtained from this experiment (i.e., the apparent equilibrium constant, K, and the enthalpy change of the binding reaction, ΔH) are listed in **Table 2**.

The values of K and ΔH for the free enzyme listed in **Table 2** were obtained either by batch calorimetry or flow isothermal titration calorimetry. The flow calorimeter is designed to measure the heat effects by mixing two liquids. Instead, batch calorimetry can easily monitor heat effects with heterogeneous samples conveniently titrated with an external liquid reactant solution. The heat of reaction is observed as long as reaction sites are present on the material inside the cell. As can be seen in **Table 2**, there is a good agreement between batch and flow techniques in the case of the binding reaction with the free enzyme. Moreover, immobilized RNase is still fully competent to bind the inhibitor, since the apparent equilibrium constant, K, and ΔH are quite close to those of the free enzyme.

Spectroscopic studies confirmed the results showed in **Table 2** for five-carbon spacer arms,C_5, or longer , up to C_{22} ***(13)***, whereas a dramatic decrease in binding affinity was instead observed for shorter-linking bridges (<C_5) ***(13)***.

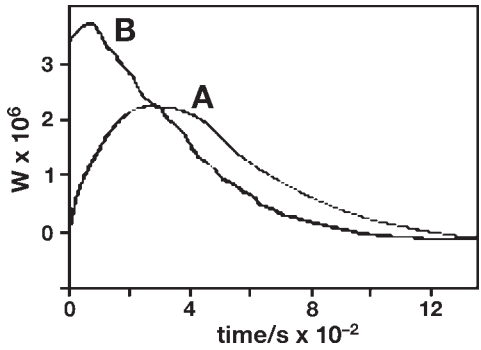

Fig. 4. Binding of 3'-CMP to immobilized RNase on CPC silica beads activated with glutaraldehyde. Acetate buffer 50 m*M*, pH 5.0. Immobilized RNase: 0.52 mg, 3'CMP titrating solution: 1.5 m*M*. Flow rate: 1.2 µL/s. *(A)* power vs time experimental output. *(B)* Curve obtained after correction of the time response delay of the instrument. (From **ref. *18***. Copyright 2005, with permission from Elsevier.)

Table 2
Apparent Equilibrium Constant (K) and Enthalpy Change (ΔH) of the Binding of 3'-CMP to RNaseA

Conditions	K ($mol^{-1} \times 10^{-4}$)	ΔH (kJ/mol)	N
Free (flow)	5.06 ± 0.1	37.4 ± 0.5	1.01
Free (batch)	5.05 ± 0.1	38.7 ± 0.5	
Immobilized on CPC (batch)	7.6 ±0.6	35.6 ± 1.0	

Acetate buffer 50 m*M*, pH 5.0. Flow and batch notations are referred to flow and batch calorimetry, respectively. *N* is the number of the binding sites. (From **ref. *18***. Copyright 2005, with permission from Elsevier.)

1.3. Adsorption: Thermal Unfolding

1.3.1. DSC Results

Inert supports can be used as protein and enzymes carriers for particular applications such as reactions in non aqueous solvent systems. Depending upon the characteristics of the support, the adsorption interactions may be based on weak interaction forces, either electrostatic or of London type (i.e., van der Waals, dipole–dipole, hydrophobic). Celite is an essentially uncharged diatomaceous earth material characterized by low water-affinity. Unlike other common adsorbing material, such as synthetic polymeric resins, celite does not induce any major structural modification on adsorbed RNase ***(23)***. The thermal unfolding of RNase adsorbed on celite in the presence of dodecane as solvent is shown in **Fig. 5A** at a water content of 0.48 gram per gram (g/g) of protein. As can be seen, the melting transition is clearly observed in these conditions. The transition strongly depends upon

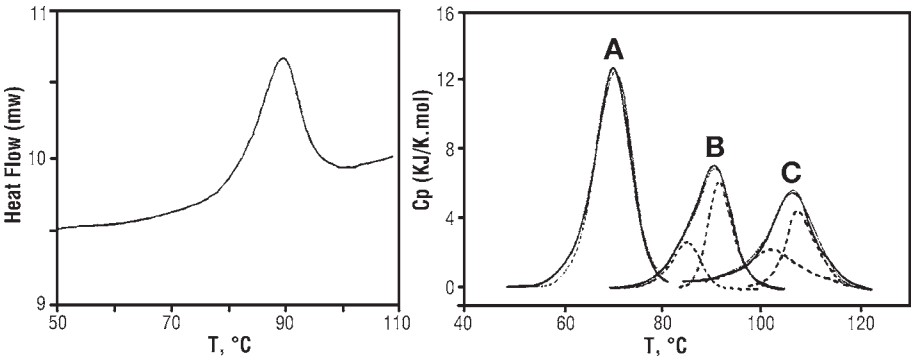

Fig. 5. DSC thermogram of the unfolding of RNase adsorbed on celite. *(A)* Direct calorimetric output in the presence of dodecane, water content 0.48 g/g. *(B)* Excess heat capacity as a function of the temperature in the presence of dodecane, water content 1.1 g/g *(A)*, 0.48 g/g (b), 0.22 g/g *(C)*. (From **ref. *25***. Copyright 2005, American Chemical Society.)

Table 3
Thermodynamic Parameters of RNase Unfolding in Phosphate Buffer 10 m*M*, pH 7.0

Conditions	H_2O (g/g)	T_{m1} (°C)	ΔH_1 (kJ/mol)	T_{m2} (°C)	ΔH_2 (kJ/mol)	ΔH_{tot} (kJ/mol)
Free (buffer)	—	65.9	480	—	—	480
Free + celite (buffer)	—	66.4	470	—	—	470
Immobilized (no solvent)	1.4	68	466	—	—	466
Immobilized (dodecane)	1.4	69.3	503	—	—	503
Immobilized (dodecane)	0.48	85.4	253	89.9	165	418
Immobilized (dodecane)	0.24	101.6	206	108	167	373

Note: parameters are the same as in **Table 1**.

hydration level, as shown in **Fig. 5B**, where the molar excess heat capacity (obtained from the raw data of **Fig. 5A** after baseline subtraction) is plotted as a function of the temperature at different water content in the presence of dodecane. As the fraction of water decreased, the transition shifted to higher and higher temperature, showing a remarkable increase of the protein stability.

From the integration of the calorimetric excess heat capacity of the DSC transition, the enthalpy change of unfolding, ΔH, is obtained. This parameter, as well as the middle point transition temperature, T_m, are listed in **Table 3**, which shows that the presence of celite itself in an aqueous environment (line 2) does not alter the thermal stability of the enzyme. Control experiments showed that the enzyme desorbed completely from the support in an aqueous solvent. Immobilization at low degree of hydration without any solvent brings about a stabilization effect, as

both T_m and ΔH increase. This effect is even more enhanced in the presence of dodecane. The progressive decrease of the water content induces a shift of the T_m to higher and higher temperature, even above 100°C. A significant progressive and concurrent decrease of ΔH is also observed. These effects may be explained by considering that at low water content the protein molecule is more "rigid" than in pure aqueous solvent. At low hydration level, water molecules cannot act any longer as a molecular "lubricant," as indeed is the case when the protein is fully hydrated ***(24)***. As a result, the overall transition splits into two components, as shown in **Fig. 5**. This probably results from domain decoupling induced by the subtle interaction between protein macromolecule and support surface and/or by the accompanying uneven dehydration of the protein macromolecule ***(25)***. Decoupling of the protein domains as a function of hydration resembles what was observed in the case of covalently linked macromolecule (*see* **Subheading 2.**).

2. Materials

2.1. Single-Point Attachment: Thermal Unfolding

2.1.1. Activation of Silica Beads

1. Controlled pore carrier (CPC)-silica beads, aminopropyl derivative, pore size 500 Å, 30–45 mesh (Fluka, www.sigmaaldrich.com), 10 g (dry weight; *see* **Note 1**).
2. Washing buffer: phosphate buffer 100 m*M*, pH 7.0.
3. 10% (v/v) Aqueous glutaraldehyde (di-aldehyde) solution.

2.1.2. Protein Immobilization

1. Protein: ribonuclease A (RNase, Sigma, Type XII A, molecular weight [MW] 13.700).
2. Immobilization buffer: 100 m*M* phosphate buffer, pH 7.0.
3. Glutaraldehyde activated CPC silica beads.

2.1.3. Number of Linkages Between Protein and Support

1. Protein: ribonuclease A RNase (Sigma, Type XII A).
2. Buffer: 100 m*M* sodium phosphate, pH 7.0.
3. Aqueous valeraldehyde (monoaldehyde) solution: 10% (v/v).
4. Solution of trinitrobenzenesulfonic acid, according to standard literature conditions ***(15)***.

2.2. Single-Point Attachment: Binding Studies

2.2.1. Calorimetric Titration Study

1. CPC-immobilized protein: 0.25 g, typically containing approx 5 mg (0.36 µmoles) of protein (*see* **Subheading 2.3.2.**).
2. Buffer: 50 m*M* sodium acetate, pH 5.0.
3. Ligand solution: 3'-CMP inhibitor solution 1 m*M* in the same buffer (10 µmoles). A 30-fold excess concentration is largely enough.

2.3. Adsorption: Thermal Unfolding

2.3.1. Protein Immobilization and Hydration

1. Protein: ribonuclease A (RNase, Sigma, Type XII A).
2. Buffer: sodium phosphate 10 m*M*, pH 7.0.

3. Adsorbing material: celite (Fluka, type 577, particle size ≤40 µm).
4. Isopiestic equilibration: at a fixed volume ratio of mixtures of water and H_2SO_4 (minimum 96%, analytical grade). The range of volume ratio may vary from 5 to 20%, according to standard reference tables ***(26)***.
5. Labware: a large vacuum-tight dry desiccator or, for very low hydration level, a high vacuum pump; standard Karl-Fisher automatic apparatus for water content determination.

3. Methods

3.1. Single-Point Attachment: Thermal Unfolding

3.1.1. Activation of Silica Beads

1. Wash 10 g (dry weight) of CPC-silica beads with washing buffer.
2. Add 20 mL of an aqueous glutaraldehyde solution.
3. Shake vigorously for 45 min at about 0°C (ice-water bath).
4. Wash the activated beads extensively with washing buffer.

3.1.2. Protein Immobilization

1. Dissolve 30 mg of RNase in 8 mL of phosphate buffer.
2. Add 1 g of glutaraldehyde activated glass beads.
3. Shake gently for 5–7 h at room temperature, then wash thoroughly with the appropriate buffer solution.
4. During the immobilization reaction, check the decrease of the protein concentration as a function of time by measuring the absorbance at 278 nm (ε = 9800 M/cm) of the supernatant solution. Usually between 80 and 90% of the initial protein can be immobilized on the glass beads.

3.1.3. Characterization of the Immobilized Protein

The concentration of the immobilized protein can be as high as 20–25 mg/g of beads. The concentration of the immobilized RNase can be determined in three ways:

1. From the absorbance change during immobilization (*see* **Subheading 3.1.2., step 4**)
2. By amino acid analysis, after acid hydrolysis at 110°C. Control experiments showed no interference from the glass.
3. By Lowry-Folin method ***(14)***. The method can be applied after dissolving the samples (the glass beads) at pH 12.0 at 100°C

The uncertainty is ±5% for methods 1 and 2 and ±15% and for method 3.

3.1.4. Number of Linkages Between Protein and Support

The number of linkages between protein and support may be indirectly estimated by measuring the amount of protein-free amino groups still reactive after treatment of the protein with valeraldehyde, a monofunctional aldehyde. The conditions of the reaction between valeraldehyde and the free protein have to be the same as those used in the reaction between glutaraldehyde-activated glass beads and protein.

1. Dissolve 40 mg of RNase in 8 mL of phosphate buffer.
2. Add valeraldehyde, 10% (v/v) and mix thoroughly for 5 to 7 h at 25°C.

3. Titrate the remaining free amino groups with trinitrobenzenesulfonic acid, according to **ref. *15***.
4. Compare the results with an appropriate reference sample of native RNase.

In these conditions it has been found that the amount of reacted amino groups were 1.2 ± 0.4 mole/mole of protein.

*3.1.5. Calorimetry (see **Notes 2–5**)*

Highly sensitive commercial microcalorimeters are currently available. Instruments such as DAMS-4 (Pushchino Scientific Center, www.psn.ru/english), MC-2 (Microcal Inc., Northampton, MA), and Micro DSC (SETARAM, Caluire, France) are suitable instruments for protein thermal stability studies. They all employ relatively small sized cells, which can be filled with heterogeneous material, although with some practical limitations. Modern instruments such as VP-DSC (Microcal) and Multi Cell MC-DSC 4100 (Calorimetry Science Corp., Spanish Fork, UT) are more sensitive than earlier versions (1970–1990) and therefore the sample size can be considerably reduced. They are all equipped with a solid sample device. Older instruments needed larger samples because they operated with larger volume cells, more easily filled and more practical to handle.

Analysis of the thermograms according to two-state approximation and the underlying theory of protein unfolding in solution are well documented ***(16,17)***. This can be applied to immobilized proteins as well. Data processing can be also conveniently performed by commercial standard software programs such as Origin Software (Microcal). Thermal analysis and deconvolution (single component) programs are usually standard software accessories of the commercial calorimeters.

3.2 Single-Point Attachment: Binding Studies

*3.2.1. Calorimetric Titration Study (see **Notes 6–7**)*

1. Load the immobilized protein (0.25 g) into the calorimeter titration cell and add 1 mL of a suitable buffer (for example acetate buffer 50 m*M*, pH 5.0).
2. Prepare 10 mL of 3'-CMP inhibitor solution 1 m*M* in the same buffer (10 µmoles).
3. Pump the inhibitor solution at a constant rate (ranging from 0.07 to 0.3 mL/min) until the heat effect decreases to baseline values (about 20 min).
4. Prepare a second sample with the same amount of supporting material without enzyme and add the inhibitor with the same rate. This experiment is a control run. The aim is to obtain a suitable baseline. The heat effects in these conditions in the absence of enzymes are minimal.

*3.2.2. Calorimetry (see **Notes 2–5**)*

Sensitive isothermal batch calorimeters are commercially available. The results presented in **Table 2** were obtained with a Titration Activity Monitor (TAM, Thermometric, Jarfalla AB, Sweden), equipped with twin titration-cells. The VP-ITC (Microcal), a heat compensation and titration calorimeter, is a suitable instrument as well (vessel volume 1.3 mL, detection limit 0.8 µW).

Isothermal batch calorimeters are the method of choice for studying reaction with immobilized proteins. Flow instruments cannot be used because heterogeneous materials cannot be introduced accurately inside the calorimeter. Instead, batch

calorimeters can quantitatively accommodate the material to be titrated inside the instrument cell and are readily accessible to an external flowing reagent. However, batch instruments require a relatively long time for thermal equilibration and this may be a serious drawback if several samples have to be tested. On the other hand, batch calorimeters have the sensitivity to examine slow reactions and the potentiality to analyse a full protein–ligand titration curve with a single run.

Analysis of the data was implemented by standard theory and software ***(19,20)***. The theory of the Tian correction is well documented from theoretical and experimental point of view ***(21,22)***. Algorithms for the calculation of the Tian effect may be available commercially (for instance, the algorithm developed by Kirchoff, NBS, Gaithersburg, MD).

3.3. Adsorption: Thermal Unfolding

3.3.1. Protein Immobilization and Hydration

1. Dissolve 30 mg of RNase in 5 mL of phosphate buffer.
2. Add the protein solution to 1 g of celite and mix throughout to form a homogeneous slurry. Spread the paste on a wide flat glass surface of suitable dimensions (10-cm diameter or more).
3. Prepare a mixture of water and H_2SO_4 at a fixed volume ratio. The volume ratio may varies from 5 to 20%, according to standard reference tables ***(26)***. The higher the sulfuric acid concentration the lower the final water content. Twenty milliliters of solution are sufficient for the isopiestic equilibration. Use a wide open glass container such as a glass Petri dish.
4. Transfer the protein–celite slurry and the sulfuric acid–water solution into a vacuum-tight dry desiccator.
5. Allow the isopiestic equilibration to take place for a week at constant temperature (for instance at 25 ± 1°C; *see* **Note 8**). By changing the water/H_2SO_4 ratio, the total water content of the protein sample may be varied from 5 to 30% (w/w) corresponding to 0.24 to 1.4 g/g of protein.
6. For very low hydration level (<0.2 g/g) the sample can be dried under high vacuum for different time intervals.
7. Measure the final water content of the protein sample by standard Karl Fisher water titration (*see* **Notes 9** and **10**).
8. Check the concentration of RNase in the dried sample by suspending the celite powder by weight in a known amount of aqueous buffer solution (phosphate buffer 100 m*M*, pH 7.0). Mix the sample vigorously and centrifuge at 4000–5000 rpm for 1 min in an Eppendorf centrifuge 5415 or 5417. Read the absorbance at 278 nm of the supernatant (ε_{278} = 9700 mol/cm). Many proteins, including RNase, are completely released from celite in an aqueous solvent.

3.3.2. Calorimeter Experiments (see Notes 12 and 13)

1. Protein samples were directly withdrawn from the desiccator and added to the pan. This did not alter significantly the hydration level of the sample providing that only few minutes elapsed from sample withdrawal to pan sealing.
2. Load a calorimeter pan with 20–25 mg of sample (containing 2–3 mg of protein), which are sufficient to observe a good signal-to-noise ratio.

3. When needed, add 20–30 L of solvent, either aqueous or organic (anhydrous).
4. Allow the samples to equilibrate for 30 min before starting the thermal ramp. The same amount of inert support and solvent was used in the reference pan in order to obtain an automatic baseline compensation.
5. Use an appropriate scan rate. Usually a scan rate range from 1 to 10°C/h are suitable (*see* **Note 11**).

3.3.3. Calorimetry (see Notes 2–5)

The best instruments to study the thermodynamic properties of adsorbed proteins are those batch calorimeters with sealed pans as sample holders. Because the amount of the sample, usually powdered material containing the adsorbed enzyme, has to be known precisely, the most convenient method of preparation, therefore, is to weigh the sample (and solvent) directly on the pan. Otherwise, precise quantitative cell loading may be difficult with a standard calorimetric cell usually built with small entrance holes.

Another important factor is the temperature range of the instrument. The calorimeters mentioned in **Subheading 2.** were developed for studies using water as a solvent and cannot span wide temperature ranges. Conversely, batch calorimeters using pan sample holders are devoted to studying temperature effects up to several hundreds of degrees centrigrade. For instance, the results of the DSC experiments showed in **Table 3** were obtained with the Perkin Elmer DSC-7 calorimeter (Norwalk, CT), which is able to span from –170°C up to 700°C.

The Mettler Toledo DSC 822 calorimeter (Columbus, OH) is a more recent model. The standard aluminum pans contain only 10–15 μL of sample. Nevertheless, this calorimeter is a sensitive instrument, reaching a nominal resolution value <0.4 μW. Crucible type pans can be used, which can contain up to 125 μL of sample and can hold 20 bars of internal pressure.

The best fitting and deconvolution analysis were carried out according to the two-state approximation mathematical model described in the literature, as already mentioned in **Subheadings 1.2.** and **3.1.5.**

4. Notes

1. Controlled pore glass beads can be purchased in different size and pore diameter (from 700 to 1400 Å) from Fluka. They are available as neat, unmodified or chemically modified glass. There are several type of chemical derivatives, including the aminopropyl group. The density of the functional group varies with the preparation. With the aminopropyl beads derivative, pore size 500 Å, 30–45 mesh, the amount of modified aminopropyl groups can be as a high as 2 mmol/g
2. Special care has to be taken on degassing because of the heterogeneity of the samples. Careful treatment under reduced pressure or the use of extensively degassed buffer solutions is required in order to avoid the formation of air bubbles during the thermal scan.
3. Another practical difficulty with all the instruments is the determination of the exact amount of material (immobilized protein) actually used to fill the calorimeter cell. The difficulty may arise from the dimensions of the calorimetric sample cell entrance hole, which is often quite small. The best approach is to standardize the quantity before adding the material to the cell. For instance, a small glass

container (a small beeker or, better, a small funnel) may be used, whose volume is calibrated. A known amount of solid support (containing the protein) may then be routinely obtained by filling the container with a fixed amount of material. This can be easily measured by weight or volume (by referring to calibration marks on the container). Then the whole amount is poured into the calorimetric cell. The amount of material for each run ranges from 0.05 to 0.2 g of support, containing about 1.5–6.0 mg of protein.

4. Thermal scan rates may vary from 30 to 120°C/h, as in the case of the free enzymes in solution. Data collection every 10–15 s usually suffices.
5. Reference scans of sample containing only the supporting material (without proteins) are necessary, although many materials (as the glass beads) do not show any significant temperature-dependent heat effects (at least up to 100°C).
6. During the titration experiment, in order to minimize the eventual grinding of the material, switch on the stirring of the sample immediately before starting the external pump.
7. The stirring of the heterogeneous sample within the calorimetric cell is another important issue. According to our experience this is a critical parameter. It is necessary to ensure a complete and thorough mixing of the reagent, otherwise spurious results may be obtained. The stirring device offered by the manufacturer may not be fully suitable and sometimes optimization is needed. For instance, the blades of the mechanical stirrer have the highest efficiency if tilted in such a way to push the sediment in an upward motion, from bottom to top.
8. During isopiestic equilibration, at very high concentration of sulfuric acid (water content <5%) it is possible that acidic components may evaporate in the vapor phase, depending upon the temperature. The presence of volatile acidic compounds can be detected by pH indicator paper added inside the dessicator. In this case it is necessary to pay attention to the possible alteration of the protein sample.
9. Most of the water present in the sample after isopiestic equilibration is used up to hydrate the protein molecules. In fact, control experiments showed that celite itself retains only a very small amount of water when equilibrated in the same conditions without protein.
10. When working with volatile solvents it is necessary to use high-pressure pans during the calorimeter run. These pans are usually provided by the manufacturer as accessories. The experiments with RNase were performed with capped stainless steel pans with an internal volume of about 80 μL, resistant to inner pressures as high as 25 bars.
11. In the case of RNase, which has a well characterized and reversible unfolding transition, the scanning rate was as high as 10°C/min. The thermodynamic parameters of unfolding were independent of the scanning rate from 5 to 15°C/min.
12. Note that, although the Perkin Elmer DSC-7 calorimeter is rather an old model, it functions on a true calorimetric principle (i.e., the power compensation "null-balance" principle). This means that the energy absorbed or evolved by the sample is compensated by addition or subtraction of an amount of electrical energy equivalent to the sample pan holder with respect to the reference pan holder. The energy and not a temperature difference is the direct output of the instrument.

13. The Mettler Toledo DSC 822 calorimeter (Columbus, OH) is a recent model. Unlike Perkin Elmer calorimeters, this instrument is based on the principle of measuring the temperature difference between sample and reference cells. Nevertheless, the sensitivity is as high as 0.4 μW.

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27

Use of Immobilized Biocatalysts in Fluidized Bed Format

Ipsita Roy and Munishwar N. Gupta

Summary

In a fluidized bed reactor, the substrate solution is fed from the lower end of the bed at a velocity high enough to lift the biocatalyst containing beads. Such reactors are especially valuable when the substrate solution contains particulate matter. The mass transfer in a fluidized bed is better as compared with packed beds. For optimum interaction between the biocatalyst and the substrate, the fluidization velocity needs to be optimized. This illustrative protocol describes a somewhat novel concept in which preparations of glucoamylase and pullulanase were entrapped in different populations of calcium alginate beads. These two entrapped enzyme preparations were premixed in the desired ratio to form a fluidized bed reactor that was used for hydrolysis of starch to glucose. The approach is different from coimmobilization because it allows one to blend the two enzymes in different ratios for optimum bioconversion.

Key Words: Bioconversion; calcium alginate beads; coimmobilization; entrapment; expanded beds; glucoamylase; pullulanase; pullulan hydrolysis; starch hydrolysis.

1. Introduction

Immobilized biocatalysts can be used in various kinds of reactors. Some examples are the batch reactor, continuous-flow stirred tank reactor (CSTR), packed bed reactor, and fluidized bed reactor. For an overview of design, operation, and chemical engineering aspects of fluidized bed reactors, the review by Godia and Sola ***(1)*** may be consulted. In a fluidized bed reactor, the substrate solution is fed from the lower end of the bed at a velocity high enough to lift the particles (*see* **Fig. 1**). Such reactors are especially valuable when the substrate solutions contain particulate matter. The mass transfer in a fluidized bed is better as compared with packed beds ***(2)***. The bed expansion during fluidization is dependent on the nature of support, the distributor design, the fluidization velocity, and the viscosity of the feed.

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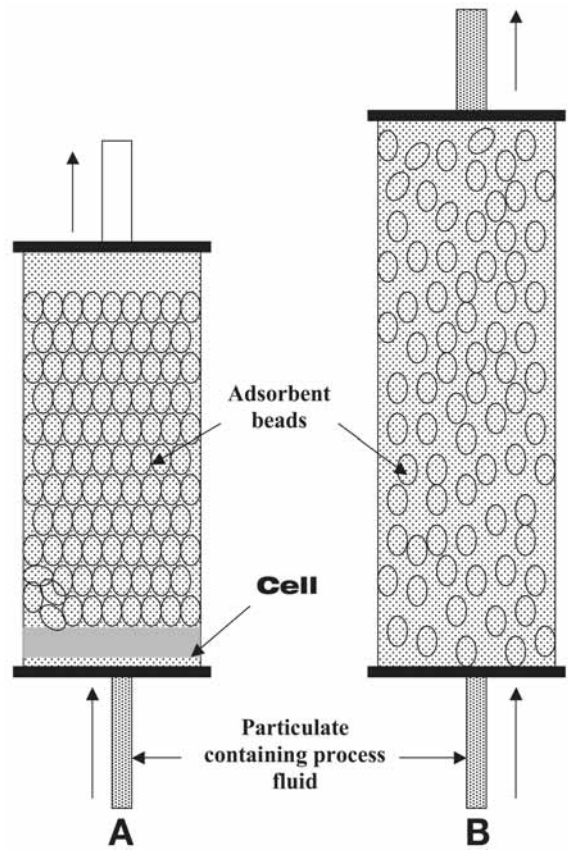

Fig. 1. (**A**) Flow of liquid through a packed bed and a (**B**) fluidized bed column.

For optimum interaction between the biocatalyst and the substrate, the fluidization velocity needs to be optimized.

Fluidized bed reactors utilizing both immobilized enzymes and whole cells have been reported (**Table 1**). Hydrolysis of tributyrin emulsion droplets by immobilized lipase in a fluidized bed bioreactor is perhaps one of the earliest reports of this approach ***(3)***. In the hydrolysis of phenylalanine methyl ester by chymotrypsin, fluidized bed reactor was found to give higher conversion rates than CSTR ***(4)***. The first pilot plant for whey hydrolysis used a fluidized bed consisting of *Aspergillus niger* lactase adsorbed on porous alumina and cross-linked with glutaraldehyde ***(5)***. More recently, whey lactose hydrolysis of Lactozym™ immobilized on cellulose beads in batch and fluidized bed modes were compared ***(6)***. The fluidized bed hydrolyzed whey lactose (<90% conversion) in 5 h as compared with 48 h required in the batch mode. The immobilized enzyme could be reused three times without any change in performance of the fluidized bed reactor. The fluidized bed could also hydrolyze milk lactose up to 60% within 5 h. Sun et al. ***(7)***

Table 1
An Illustrative List of Reactions Catalyzed by Immobilized Systems on Fluidized Beds

Protein/cell	Matrix	Immobilization method	Reaction catalyzed	Ref.
β-Galactosidase	Cellulose beads	Epichlorohydrin activation	Hydrolysis of whey lactose	6
Catalase	Chemically modified DEAE-cellulose	Adsorption	Decomposition of H_2O_2 in pasteurized milk	13
Escherichia coli	Ca-alginate beads	Entrapment	Reduction of nitrite	14
Yeast cells	Ca-alginate beads	Entrapment	Study of diacetyl levels in green beer	15
Streptomyces tendae	Sintered glass	Adsorption	Production of nikkomycin	16
Yeast cells	Porous glass beads	Adsorption	Accelerated fermentation system	17
Penicillium janthinellum P9	Polyurethane	Adsorption	Production of chitinolytic enzymes	18
Rhodococcus spp.	Ca-alginate beads	Entrapment	Conversion of 19-nortestosterone steroid to estrone	19
Zymomonas mobilis	κ-Carrageenan	Entrapment	Fermentation of glucose to ethanol	8
Bacillus megaterium	Polyacrylamide hydrazide gel	Entrapment	Hydroxylation of 15β-steroid	20
Pseudomonas putida	Ca-alginate beads	Entrapment	Biodegradation of phenolic wastewater	21
Saccharomyces diastaticus	Polyurethane	Entrapment	Bioconversion of acetanilide to *p*-acetaminophene	22
Methylocystis spp.	Ca-alginate beads	Entrapment	Biodegradation of trichloroethylene	23
Pseudomonas putida	Ba-alginate beads	Entrapment	Biotransformation of toluene to *cis*-diol	24
Glucoamylase	Corn stover	Covalent grafting	Hydrolysis of maltodextrin	25
Lipase	Stainless steel	Adsorption	Hydrolysis of tributyrin	3
Chymotrypsin	Acrylamide	Covalent coupling	Stereospecific hydrolysis of D,L-phenylalanine methylester	4
Gluconobacter oxydans	Ca-pectate beads	Entrapment	Bioconversion of glycerol to dihydroxyacetone phosphate	26
Humicola spp.	Acrylamide	Copolymerization	Biotransformation of Rifamycin B to Rifamycin S	27
Cyclodextrin-glycosyl-transferase	Controlled pore silica	Adsorption	Production of cyclodextrin	28
Arthrobacter simplex	Polyacrylamide hydrazide gel	Entrapment	Dehydrogenation of L-hydrocortisone	29
Klebsiella pneumoniae	Alginate	Entrapment	Removal of mercury from wastewater	30

evaluated the performance of fluidized bed reactor consisting of immobilized *Saccharomyces cerevisiae* and amyloglucosidase for bioconversion of soluble starch and yeast extracts. The biocatalyst exhibited no significant loss of activity during many weeks of continuous operation. Recently, Krishnan et al. ***(8)*** have looked at the economics of fuel ethanol production from cornstarch. The glucose fermentation step was carried out in a fluidized bed reactor using *Zymomonas mobilis* entrapped in κ-carrageenan beads. It was estimated that an operating cost saving in the range of 1.1–1.3 cents/gal was possible with fluidized bed reactor technology for a 15-million gal/yr ethanol plant.

In another approach, it has been found that if the bed consists of magnetically susceptible particles, application of a magnetic field facilitates control of fluidization. In such magnetically stabilized fluidized beds, a weak stable external magnetic field is applied axially, relative to the flow of the fluidizing medium. This makes it possible to operate at higher fluidization velocities. Webb et al. ***(9)*** have provided a good discussion on the advantages of such beds with a focus on improved mass transfer studied with a number of immobilized enzyme systems.

The protocol described in this chapter illustrates a somewhat novel concept in which preparations of two different enzymes, each immobilized on the same matrix, were premixed to carry out a sequential conversion in the fluidized bed reactor.

2. Materials

2.1. Assay of Pullulanase and Glucoamylase

1. Pullulanase from *Bacillus acidopullulyticus* (supplied as Promozyme, Novozymes®, Krogshoejvej, Denmark).
2. Glucoamylase from *Aspergillus niger* (supplied as Palkodex, Maps India Ltd., Ahmedabad, India).
3. Soluble starch from potato (E. Merck, Mumbai, India).
4. Pullulan (Sigma Chemical Co., St. Louis, MO).
5. Buffer 1: 0.05*M* sodium acetate buffer, pH 5.0.
6. Buffer 2: 0.05*M* sodium acetate buffer, pH 4.5.
7. Dinitrosalicylic acid reagent ***(10)***.

2.2. Entrapment of Glucoamylase and Pullulanase in Alginate Beads

1. Sodium alginate (low viscosity, Sigma).
2. Small magnetic bar.
3. Magnetic stirrer.
4. Hand-held disposable syringe, with a volume of 20 mL.
5. 22-Gauge needle.
6. 1 *M* $CaCl_2$ solution.
7. Buffer 3: Buffer 1 containing 0.006 *M* $CaCl_2$.

2.3. Hydrolysis of Starch in the Fluidized Bed Mode (see Note 1)

1. Jacketed glass column with a frittered end (1 × 20-cm column; Sigma).
2. Water bath with circulator.
3. Flow adapter (for 1-cm inner diameter column; Sigma).

4. Peristaltic pump with flow rates ranging from 0.5 to 20 mL/min (Alitea AB, Sweden).
5. Frac 100 fraction collector (Pharmacia, Uppsala, Sweden).

3. Methods

3.1. Assays of Pullulanase and Glucoamylase

1. Incubate 0.5 mL pullulanase, appropriately diluted in Buffer 1 in a reaction mixture containing 0.4% pullulan in 0.5 mL Buffer 1 at 40°C.
2. Stop the reaction after 30 min by adding 1 mL dinitrosalicylic acid reagent ***(10)*** and immersing the test tubes in a boiling water bath.
3. Cool the test tubes after boiling for 5 min, add 10 mL of distilled water, shake, and read the absorbance of the liberated reducing sugars at 540 nm. One enzyme unit liberates 1 µmol of glucose/min at 40°C, pH 5.0.
4. Incubate 0.5 mL glucoamylase, appropriately diluted in Buffer 2, in a reaction mixture containing 1% starch in 0.5 mL Buffer 2, at 65°C. Stop the reaction after 15 min by adding 1 mL dinitrosalicylic acid reagent ***(10)*** and proceed as in **step 2**. One enzyme unit liberates 1 µmol of glucose/min at 65°C, pH 4.5.

*3.2. Entrapment of Pullulanase and Glucoamylase in Alginate Beads (***see *Note 2)***

1. Add 2.5 g alginate to 100 mL Buffer 1 and stir on a magnetic stirrer till the polymer dissolves. Add 4536 U/mL pullulanase to 15 mL of alginate solution and stir gently on a magnetic stirrer until a homogeneous solution is formed.
2. Fill a syringe with 20 mL of this solution and add it dropwise through a needle into a 100-mL solution of 100 mL $CaCl_2$ so that beads are formed. Store the beads in this solution for 1 h and then transfer to Buffer 2 containing 0.006 *M* $CaCl_2$.
3. Wash the beads repeatedly with this solution until no enzyme activity is detected in the washings.
4. The beads were kept at 4°C until further use. 458 U of glucoamylase was entrapped in a similar manner (*see* **Note 3**).

3.3. Hydrolysis of Starch in the Fluidized Bed Mode

1. Pack a jacketed glass column with enzyme-entrapped alginate beads (127.5 U of glucoamylase and 85 U of pullulanase; *see* **Note 4**) and wash the beads thoroughly with Buffer 3.
2. Maintain a water bath at 45°C and circulate the water around the jacketed column. Connect tubings from the peristaltic pump to the bottom of the column.
3. Fix the adapter at the top of the column and connect the tubings out of it to the fraction collector (*see* **Fig. 2**).
4. Adjust the height of the flow adapter so that there is enough space on top of the bed for the beads to move upwards.
5. Pump the equilibrating buffer to the bottom of the column and note the increase in height of the settled bed of beads containing entrapped enzymes.
6. When the bed has fluidized to the desired height (*see* **Note 5**), pump in 1% starch from the bottom of the column at a flow rate of 2 mL/min, and collect the hydrolyzate in a fraction collector (*see* **Note 6**).

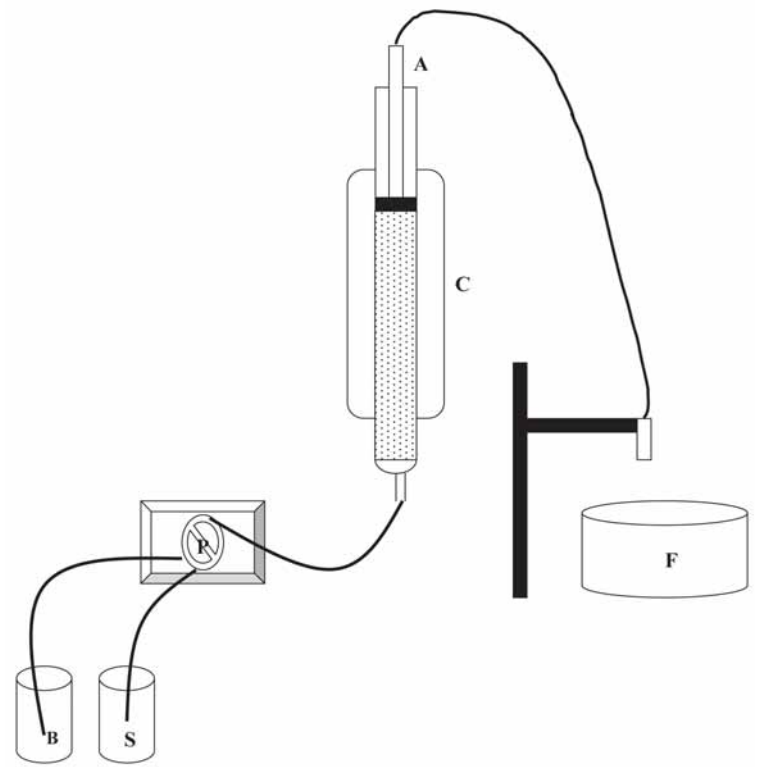

Fig. 2. Schematic diagram of starch hydrolysis (S) on a fluidized bed column (C) of entrapped glucoamylase and pullulanase. Flow adapter (A), fraction collector (F), peristaltic pump (P), Buffer 3 (B).

4. Notes

1. Fluidized beds inherently have large void volumes. Hence high biocatalyst concentration (per unit volume of the reactor) is not possible.
2. In general, calcium alginate beads are not used for enzyme entrapment as the large pore sizes of these beads result in enzyme leakage out of these beads. However both glucoamylase and pullulanase are known to bind to alginate *(11)*. Thus, presumably, in both cases, it is a combination of binding and entrapment that keeps the enzyme firmly held in each case.
3. The approach illustrated here is possible only if the same beads are used for the immobilization of two (or more!) enzymes (on different populations of beads!). If the beads used in the two cases are different, their fluidization behavior will be different.
4. This approach is different from two enzymes coimmobilized on the same beads. The strategy described here allows one to vary and determine optimum blend of the two immobilized enzymes in a facile manner.

5. Fluidized beds, which are "stable" and where backmixing is very low, have been termed "expanded beds" in the context of bioseparation. For a good discussion on the theory and operation of expanded beds, please consult ref. *12*.
6. Comparative data about performance of fluidized bed and other reactors is rather meager. Hence it is advisable that one explores all reactor designs for process optimization. For substrate solutions containing suspended matter, fluidized bed reactors offer the clear advantage of working with such feed without any preclarification.

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28

Taylor–Couette Vortex Flow in Enzymatic Reactors

Roberto Campos Giordano and Raquel de Lima Camargo Giordano

Summary

Taylor vortex flow reactors (VFRs) are especially useful when dealing with fragile biocatalysts, in view of the low-shear stress that is a characteristic of this flow pattern. Hence, they may be an interesting solution for reactions with immobilized enzymes. This chapter presents some basic features of this reactor. It is not intended to provide a review of the extensive literature on Taylor vortex flow reactors; rather it includes some of the available correlations that may be useful to design enzymatic VFRs for specific applications. Some clues concerning reactor operation that may help in everyday laboratory practice are highlighted, too.

Key Words: Enzymatic reactor; fragile supports; shear stress; Taylor vortices; vortex flow reactor.

1. Introduction

The secondary flow pattern that appears above a critical rotation in the gap between an inner (rotating) and an outer (generally, stationary) cylinder is named Taylor vortex flow ***(1)***.

One important reason for using vortex flow in biochemical reactors is its ability to promote a gentle, but still efficient, stirring, which is ideal when dealing with fragile cells (e.g., mammalian cells) or with shear-sensitive particles, such as gel matrices for immobilized enzymes (*see* **Note 1**). Another advantage of this system is that the rotation of the inner cylinder provides an additional degree of freedom for the operation of the reactor, which facilitates the fluidization of the biocatalyst.

Taylor vortices are probably one of the most widely studied phenomena in the field of fluid dynamics, ever since Taylor, in 1923, solved Navier–Stokes equations for the disturbances of Couette flow (disregarding the nonlinear terms) and predicted the onset of the vortices ***(2)***. In the same paper, experimental results supported the theoretical predictions with striking accuracy. Above a critical rotation of the inner cylinder, counter-rotating toroidal vortices appeared, superim-

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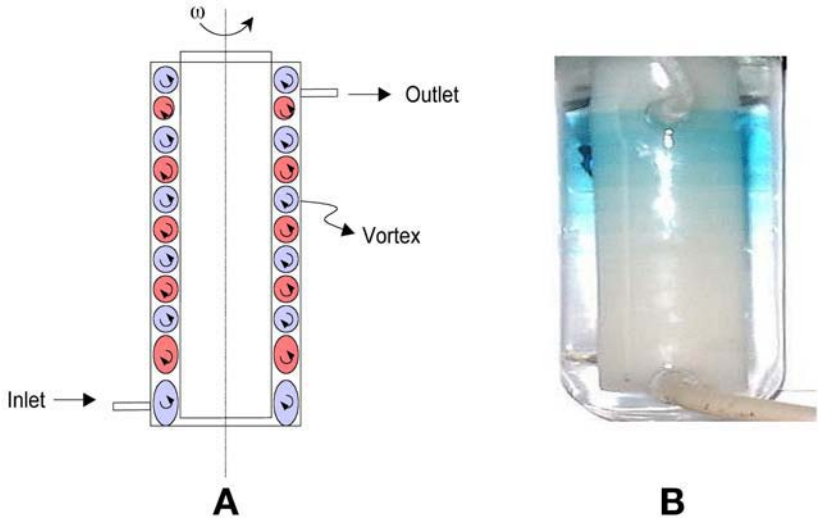

Fig. 1. Continuous vortex flow reactor.

posed to the main Couette flow. Furthermore, the size of the vortices agreed with Taylor's calculations. Chandrasekhar ***(3)*** provides an excellent formalization of the linear solution of this problem.

This chapter does not intend to provide a revision of the extensive literature on Taylor vortex flow; for instance, Tagg ***(4)*** presents approx 1500 selected references on this topic. Instead, only the main aspects that might be useful from a practical viewpoint, for a possible application of Taylor vortex flow reactors (VFRs) with immobilized enzymes, are addressed here.

Enzymatic VFRs may operate either in continuous or in batch mode. In the first case, an axial Poiseuille flow is forced through the apparatus. **Figure 1A** schematically depicts a continuous VFR. **Figure 1B** shows a picture of it after the injection of a tracer.

2. Vortex Flow Bioreactors

Taylor vortices have been used to enhance the performance of bioreactors. Batch cultures of plant cells ***(5)*** and the continuous operation of photocatalytic processes ***(6)*** are two examples of cultivation processes using VFRs. Taylor flow is also used in downstream operations such as ultrafiltration ***(7)*** and adsorption ***(8)***.

When working with immobilized enzymes, a possible alternative would be immobilization on the inner cylinder wall ***(9)***. This option could overcome a limitation that the fluidization of particles imposes (i.e., a lower limit for rotation rates). Despite this advantage, that configuration has not prevailed, probably because of its lower biocatalytic area. Hence, fluidized bioparticles, inserted in the annular gap, are the usual choice for enzymatic VFRs (*see* **Note 2**). Some processes where this configuration was employed are cleavage of heparin with immobilized heparinase in extracorporeal devices ***(10–12)***, glucose–fructose isomerization ***(13,14)***, casein hydrolysis ***(15)***, and cheese whey protein hydrolysis ***(18,20)***.

Notation

Ar	Archimedes number ($d_p^3 g\rho(\rho_p - \rho)/\nu$)
d	Reactor gap ($R_e^- R_i$), L
d_p	Biocatalyst average diameter, L
D_{ax}	Axial dispersion coefficient, L^2T^{-1}
D_i	Stirred tank impeller diameter, L
L	axial length of the reactor, L
N	rotation rate, rotations/s
P	stirring power, $ML^{-2}T^{-3}$
R_i	Inner cylinder radius, L
R_e	outer cylinder radius, L
Re_θ	rotational Reynolds number (= $\omega R_i d/\nu$)
$Re_{\theta,c}$	critical rotational Reynolds number
Re_{ax}	axial Reynolds number (= $U_{ax} d/\nu$)
Ta	Taylor number ($\omega R_i^{0.5} d^{1.5}/\nu$)
Ta_c	critical Taylor number
U_{ax}	superficial velocity (volume flow/gap cross area), LT^{-1}
Greek letters	
Γ	aspect ratio, (= L/d)
η	radius ratio, (= R_i/R_e)
μ	dynamic viscosity, $ML^{-1}T^{-1}$
ν	kinematic viscosity, L^2T^{-1}
ρ	Fluid density, ML^{-3}
ρ_p	Biocatalyst density, ML^{-3}
τ	Mean residence time, T
ω	rotation rate (rd/time), L^{-1}

The rotation rate of the inner cylinder is an important variable of operation in VFRs. Changing this speed, it is possible to span a series of different flow patterns, ranging from an almost plug-flow to an almost perfectly mixed continuous stirred tank reactor (CSTR) ***(16)***. This operational flexibility may be useful when the equipment is employed in multipurpose bioprocess units.

VFRs may sustain suspended particles in the gap between cylinders even for low axial flow rates (i.e., high residence times); conventional fluidized bed reactors would need a recycle in this situation. Another interesting aspect for further exploration is the use of varying cross-sections, for instance, in conical units when the medium viscosity changes significantly along the reactor.

Figure 2 shows start-up results of a VFR running the tailor-made hydrolysis of cheese whey proteins ***(17–21)***, using Alcalase® (Novo Nordisk, Bagsvaerd, Denmark) immobilized on glyoxyl–agarose. As shown in **Fig. 1**, the VFR was a jacketed glass vessel (working volume 2.46×10^{-4} m^3), with a polypropylene inner cylinder, radius ratio η = 0.48, and aspect ratio, Γ = 11.9 (*see* **Table 1** for the definition of these quantities).

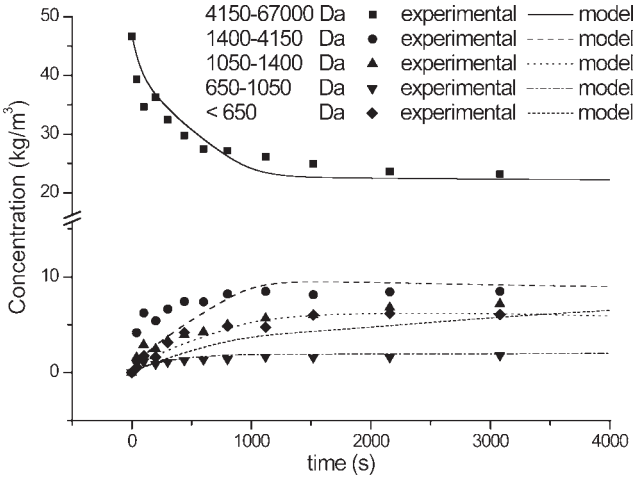

Fig. 2. Start-up of a continuous enzymatic VFR. Proteolysis of concentrated cheese whey with alcalase/agarose, 50°C (pH 9.0), mean residence time = 1800s. Product lumped in four ranges of peptides' molecular weights.

Table 1
Characteristic Dimensionless Quantities in Taylor–Couette–Poiseuille Flow

Rotational
Re_θ Rotational Reynolds number $\left(\frac{\omega R_i d}{\nu}\right)$
$Re_{\theta,c}$ Critical rotational Reynolds number for the onset of Taylor vortices
Ta Taylor number $\left(\frac{\omega R_i^{0.5} d^{1.5}}{\nu}\right)$ among several other definitions
Ta_c Critical Taylor number for the onset of vortex flow
Axial
Re_{ax} axial Reynolds number $\left(\frac{U_{ax} d}{\nu}\right)$
Geometry
η radius ratio (R_i/R_o)
Γ Aspect ratio (L/d)

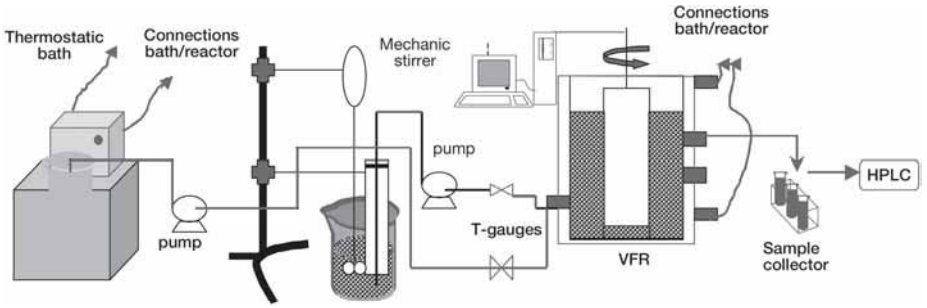

Fig. 3. Experimental apparatus for VFR enzymatic assays.

Rotation rates and reaction temperature (50°C) were controlled by a T&S (São Carlos, Brazil) computer interface unit, with accuracy of 0.1 s^{-1} and 1°C, respectively. Bed porosity was 98%. The biocatalyst was agarose gel, loaded with 7.8×10^3 U_{BAEE}/kg_{gel} (1 U_{BAEE} is the amount of Alcalase that hydrolyzes 1.0 μmol of benzoil arginine ethyl ester (BAEE)/min at pH 8.0 and 25°C). The enzyme was covalently bonded to porous particles of agarose–glyoxyl gel 6% (weight basis; Hispanagar, Spain) through multipoint links ***(18)***. The substrate was 50.0 kg/m^3-sweet cheese whey (proteins weight fractions: β-lactoglobulin 56%; α-lactoalbumin 20%; bovine serum albumin [BSA] 11%, immunoglobulins and other fractions 13%), provided by Cooperativa de Laticínios (São Carlos, Brazil). Proteolytic activities of the biocatalyst were always checked before and after each reaction assay. Enzyme losses from the biocatalyst were negligible in all assays, proving the stability of the multipoint attachments. **Figure 3** schematically depicts the experimental apparatus used to perform VFR cheese whey enzymatic hydrolysis assays.

All assays had $Re_\theta = 3500$ and $Re_{ax} = 1.10$. Concentrated cheese whey (50.0 kg/m^3), with 0.25 *M* sodium tetraborate and 0.1 *M* NaOH was fed to the reactor from a thermostatic bath (Neslab) at 50°C. The pH was adjusted to 9.5 and 2.20×10^{-4} m^3 of this solution was used in the jacketed reactor. The inner cylinder rotation was 20.9 s^{-1}. Biocatalyst beads were maintained in 0.25 *M* sodium tetraborate/1.0 *M* NaOH buffer solution at pH 9.5, with 17% volume particles/volume buffer, under stirring. At the beginning of the assay, the gel was added to the reactor (up to 2% v/v). The feed of gel (17% v/v) started immediately, to replace the loss at the VFR outlet, which was recycled to the reactor (*see* **Note 3**). The mass of gel in the VFR was measured at the end of the assay, and the steady-state regime was confirmed. Samples were analyzed through HPLC, according to the procedure previously described ***(17)***. Five markers were used to define the molecular weight distribution (MWD) intervals of the products: BSA (67,000 Da), β-lactoglobulin (18,000 Da), insulin (5000 Da), angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) (MW 1046.7 Da), and leucine enkephalin (Tyr-Gly-Gly-Phe-Leu) (555.6 Da).

The next subheadings summarize some useful equations for the design of a VFR. It should be emphasized once again that this is by no means a comprehensive review, but a personal choice of some correlations, among several others, available in the literature.

3. Stability Criteria: The Onset of Taylor Vortices

Taylor flow patterns may be classified in laminar (immediately after the onset of the Couette flow instability), wavy (with distinct numbers of azimuthal waves), and turbulent vortices, for increasing rotation rates of the inner cylinder. When operating with biocatalyst particles, the conditions for minimum fluidization generally impose the operation in the region of turbulent vortices, for biocatalysts slightly denser than the medium.

In practice, the bioreactor designer must calculate the critical rotation rate, either for closed (batch reactors) or for open systems (continuous reactors, in the Taylor–Couette–Poiseuille [TCP] regime). Different authors use different dimensionless numbers to describe Taylor flow, depending on the formalism employed in the solution of the Navier–Stokes equations. **Table 1** shows one possible set of dimensionless quantities that describe TCP flow. Two options are given for the dimensionless number that takes into account the rotation of the inner cylinder: the tangential Reynolds number (Re_θ), and one of the definitions of the Taylor number (Ta); any of them may be used without any loss of generality.

The critical rotational Reynolds number for a closed VFR may be predicted by Esser and Grossman ***(22)*** equation:

$$\mathrm{Re}_{\theta,c} = \frac{1}{0.1556^2}\frac{(1+\eta)^2}{2\eta\sqrt{(1-\eta)(3+\eta)}} \tag{1}$$

Generally speaking, when $\Gamma > 15$ end effects are not significant. Because most of the bioreactors comply with this restriction, the equations transcribed here do not take into account the aspect ratio of the apparatus.

The superposition of an axial flow to this system, necessary for a VFR in continuous operation, induces a stabilization of the Couette flow, delaying the appearance of vortices. The equation of Recktenwald et al. ***(23)*** estimates the critical Ta number for a continuous VFR:

$$Ta' = a_0\left[1+\left(\frac{\mathrm{Re}_{ax}}{a_2}\right)^2+\left(\frac{\mathrm{Re}_{ax}}{a_4}\right)^4\right], \text{with } Ta' = \frac{1-\eta}{\eta}\left(\frac{\omega R_i d}{\nu}\right)^2 \tag{2}$$

Parameters a_i may be found in reference ***(23)***, as a function of η. Notice the different definition of the Taylor number (here named Ta') adopted by the authors.

It should be stressed that the effect of the axial flow on the onset of the instability is negligible for typical values of the superficial velocity (U_{ax}) in enzymatic reactors (i.e., for mean residence times around 10 to 30 min).

When the rotation rate of the inner cylinder increases, turbulent Taylor vortices arise. This is an interesting example of a complex system: the flow in this regime is locally random, but there is still a macroscopic degree of order, since the vortex structure remains present up to very high rotations. Di Prima and Swinney's ***(24)*** review reports that the full outcome of turbulence occurs above $Re_\theta/Re_{\theta,c} \approx 25$ (for an apparatus with $\eta = 0.875$).

4. Particle Fluidization

In a heterogeneous VFR, with particles composed by the usual matrices for enzyme immobilization (for instance, agarose, chitosan, silica), dragged by the

vortices in a typical medium (not too viscous), the minimum rotation for uniform fluidization (*see* **Note 4**) will generally be high enough to ensure turbulent vortices. Resende ***(20)*** has fitted an empirical correlation for the minimum fluidization conditions, when bed porosity is in the range 0.92–0.98:

$$\mathrm{Re}_{\theta,p} = \mathrm{Re}_{\theta}\frac{d}{d_p} = 42.2Ar - 16.7\eta \tag{3}$$

for $0.48 < \eta < 0.66$ and $7.1 < Re_{\theta,p} < 75.6$. *Ar* is the Archimedes number:

$$Ar = \frac{d_p^3 g\rho\left(\rho_p - \rho\right)}{v^2}$$

5. Mass Transfer

Mass and heat transport to the walls of a Taylor device have been thoroughly studied. For the sake of concision, however, these phenomena will not be addressed here, because VFRs with immobilized enzymes generally work with suspended particles. Hence, the most important mass transfer parameters of the reactor are related to bed dispersion and to the transport through the particles' external film. Of course, intraparticle, pore-diffusion resistances may be significant, but this point is independent of the reactor configuration and therefore is out of our present scope.

Several authors have proposed macro-flow models for VFRs. Essentially, models with one, two, or three parameters are presented in the literature ***(16)***. Depending on the specific application, models that are more complex may be necessary. Nevertheless, for preliminary calculations the simplest, one-parameter approach might suffice. This is the classical axial dispersion model, and Moore and Cooney ***(25)*** presented a correlation for this parameter, spanning laminar and turbulent vortices:

$$\frac{D_{ax}}{U_{ax}L} = 7.2\times10^{-3}\left(\frac{d}{R_i}\right)^{-0.28}\mathrm{Re}_{\theta}^{1.05}\left(2\,\mathrm{Re}_{ax}\right)^{-0.83}\left(\frac{2d}{L}\right) \tag{4}$$

The authors call attention for the fact that a high load of particles may cause an increase in D_{ax} (up to twofold figures).

Increasing the rotation rates of the inner cylinder will eventually overcome film resistance and turn the inherent (or the apparent, when pore diffusion is significant) enzymatic reaction velocity into the rate-controlling step. For a typical enzymatic reaction, this will be possible within a reasonable range of rotation rates of the inner cylinder. **Figure 4** is a practical example of this situation.

6. Stirring Power

One important advantage of VFRs is low-shear stress, when compared with conventional impellers. Essentially, for moderate to high Taylor numbers, $t \propto Ta^m$, with *m* between 1.5 and 1.57 (using the definition for *Ta* shown in the **Notations** table).

Two of the published correlations for stirring power were selected here to illustrate this point. For low rotational Reynolds numbers, Dubrulle and Hersant ***(26)***

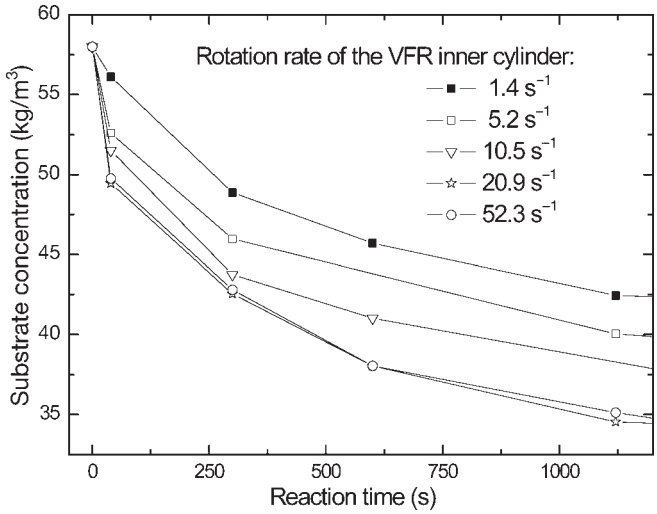

Fig. 4. Testing the presence of external resistance to mass transfer. VFR Batch assays. Bioparticle: agarose gel (55×10^{-6} m) with multipoint-attached alcalase. Substrate: cheese whey concentrate, 58 kg/m^3 (pH 9.5) and 50°C. For $\omega > 21$ s^{-1} external mass transfer resistances became negligible ***(17)***.

use an analogy with turbulent convection to calculate the torque on the inner cylinder, resulting in the following equation for the agitation power, P:

$$P = 1.46\omega\rho v^2 L \frac{\eta^{3/2}}{(1-\eta)^{7/4}} \left(\mathrm{Re}_\theta\right)^{3/2}, \text{ for } 400 < \mathrm{Re}_\theta < 10^4 \tag{5}$$

$$P = 0.50\omega\rho v^2 L \frac{\eta^2}{(1-\eta)^{3/2}} \frac{\mathrm{Re}_\theta^2}{\ln\left[\left(\frac{\eta^2(1-\eta)\mathrm{Re}_\theta^2}{10^4}\right)\right]^{3/2}}, \text{ for } \mathrm{Re}_\theta > 10^4$$

These equations were validated against experimental data within the range $400 < Re_\theta < 10^6$. For higher values of Re_θ, Moore ***(5)*** adopted the correlation of Beaudoin and Jaffrin ***(27)***:

$$P = 0.46\pi\mu Ta^{0.5} \frac{\omega^2 R_i^3}{d} L \tag{6}$$

using the definition of Ta from the **Notations** table.

It is interesting to compare the stirring power of a VFR with a conventional tank. With this purpose, the correlation of Rushton, Costich, and Everett, after Bailey and Ollis ***(28)***, was used to estimate P for a 1-m^3 baffled stirred tank, with water at 25°C, turbulent flow and a flat blade impeller (i.e., power number $P0 = 4$, and $P_{\text{stirred tank}} = P_0\rho N3D_i^5$, with N in rotations/s). The 1-m^3 VFR has $\eta = 0.618$. **Figure 5** shows the results. The stirred tank was assumed to have height equal to diameter and its impeller diameter (D_i) was set equal to the VFR inner cylinder diameter.

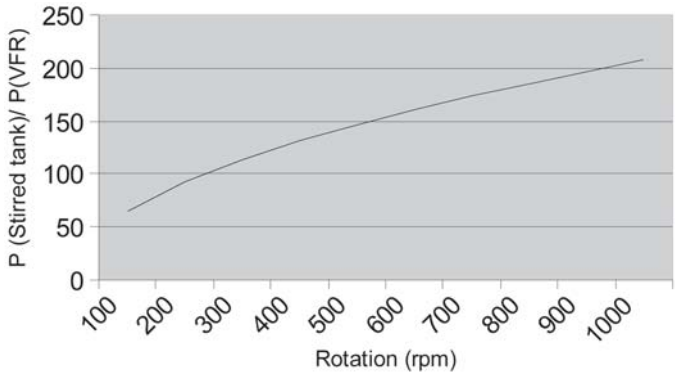

Fig. 5. Comparison between VFR and agitated tank stirring powers, for 1 m^3 reactors: P_{VFR} from **Eq. 6**.

It should be noticed that, for the VFR dimensions and fluid properties used here, $Re_\theta > 10^6$ already at 30 rpm. Therefore, the rotations in **Fig. 5** are beyond the validation range of Re_θ used for **Eq. 5**.

Equation 5 predicts that the ratio $P_{\text{stirred tank}}/P_{VFR}$ varies linearly with ω, whereas **Eq. 6** gives $P_{\text{stirred tank}}/P_{VFR} \propto \omega^{0.5}$. Anyway, the agitation power (and, consequently, shear stress) is much lower for VFRs than for conventional stirred tanks. For tanks without baffles, in turbulent regime, the ratio $P_{\text{stirred tank}}/P_{VFR}$ would be smaller, but the VFR would still have lower energy consumption.

7. Reactor Scale-Up

The fluidynamics of TCP flow, in the absence of end effects (i.e., for an infinite-length apparatus) is characterized by three dimensionless parameters: the rotational (Re_θ) and the axial (Re_{ax}) Reynolds numbers, and the radius ratio ($\eta = R_i/R_e$) of the apparatus (for an in-depth discussion of linear models of TCP flow, *see* **ref. *3***). The role of end effects is linked to a fourth parameter, the device aspect ratio ($\Gamma = L/d$). Nevertheless, VFRs usually are not sensitive to entrance/exit disturbances. Hence, the characteristic numbers Re_θ, Re_{ax}, and η should be the basis for reactor scale-up.

A rough estimate of capital costs, following Peters and Timmerhaus ***(29)***, shows that the fixed cost of a VFR (with $\eta \approx 0.65$) would be approx 18% higher than that of a CSTR with the same working volume (because of the "dead volume" of the inner cylinder). The lower power demands of the VFR, however, implies lower variable costs.

8. Notes

1. The VFR design must minimize damage of shear-sensitive particles (e.g., gel supports) in long-run experiments. One of the important causes of particle destruction is the friction between moving and stationary parts of the equipment (seals and bearings).

2. To avoid crushing of biocatalyst, the reactor should be assembled in the vertical position, and the bottom bearing of the inner cylinder should be eliminated, keeping it in balance. The outlet (for a continuous VFR) should be positioned at the cylinder wall, below the top of the apparatus (i.e., there should be a heading space, filled with air or nitrogen). In this way, fluid and particles have no contact with moving seals. The extreme vortices within the VFR always impel the particles towards the seals. If the end walls of the device are stationary, the adjacent vortex layer moves towards the inner cylinder, dragging the particles to the friction zone (the seal between the inner cylinder and the stationary wall). A drawback of this design is that the absence of the bottom bearing increases the internal cylinder wobbling, and thus an accurately balanced cylinder must be used when rotation rates should be high.
3. In continuous operation, it may be interesting to recycle the catalyst instead of entrapping it within the VFR with a sieve that might clog. When pumping a suspension of particles, clogging is common. This problem can be avoided by alternating the suspension flow with air bubbles.
4. Minimal rotations to ensure uniform fluidization in a closed device may be lower than for a continuous reactor, because entrance effects disturb the first vortex and the axial flow will be small. Accumulation of particles at the bottom of the device, close to the inlet, must be avoided; one alternative may be feeding the particles in the center of the vortex.

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29

A Novel Immobilization Method for Entrapment *LentiKats®*

Marc Schlieker and Klaus-Dieter Vorlop

Summary

Immobilization by entrapment in hydrogel particles based on polyvinyl alcohol (PVA) shows many advantages in immobilizing living cells and cross-linked enzymes. The immobilization takes place at room temperature in aqueous environment. The outstanding advantages compared to other immobilization methods are described. Subsequently the different possibilities for forming these hydrogel particles are introduced and many practical tips are given for understanding important parameters like initial biomass content or the survival rate during the immobilization step.

The benefit of this immobilization technique is shown by some examples of successful immobilization of whole cells and enzymes in LentiKats®: bioconversion of raw glycerol to 1,3-propanediol, bioethanol production from nonsterile molasses, reduction of energy consumption for sewage treatment, and production of (*R*)-cyanohydrins by using entrapped (*R*)-oxynitrilase in LentiKats.

Key Words: Hydrogel; polyvinyl alcohol; PVA; LentiKats®; whole cell immobilization; growth inside porous gel structure; enzyme entrapment; nonsterile bioconversion; 1,3-propanediol; ethanol production; (*R*)-cyanohydrin; waste water treatment.

1. Introduction

The outstanding advantages of immobilization in biotechnology impelled whole generations of scientists to search for suitable methods to use these benefits and to overcome the problems at the same time.

The main improvements are a significantly increased productivity, an easy separation that allows use in repeated or continuous processes, and a high process stability including a protection of labile cells. On the other hand, some drawbacks usually come along with an immobilization: diffusion limitation of the immobilized biocatalyst, deactivation during immobilization, and abrasion of the immobilization matrix during the use in bioreactors. Last but not least there are additional costs of immobilization.

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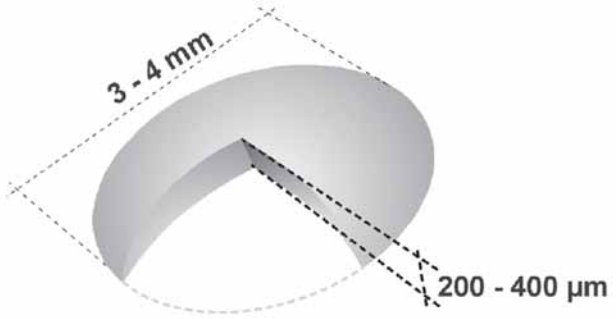

Fig. 1. Sketch of a LentiKat.

From many different immobilization methods, we chose entrapment in hydrogel particles. By preparing porous particles of natural or synthetic polymers around the biocatalyst, nearly every cell type or part of a cell can be immobilized. The most prominent examples are hydrogels of polysaccharide like carrageenan, pectate, and alginate. Hydrogel beads allow cell growth inside the gel and offer an almost optimal environment to perform bioconversions except for a low mechanical stability. High ratios of microorganisms (>30% [v/v]) or intense gas evolution can lead to bead disruption. In the case of calcium alginate beads the presence of chelators (e.g., phosphate buffer), may lead to deterioration. In addition to that the immobilization matrix is highly biodegradable.

To overcome the problems of these natural polymers and benefit from the great advantages at the same time one can switch to polyvinyl alcohol (PVA)-based matrices. PVA is a synthetic polymer that can be used to form hydrogels by the conventional freeze–thawing method and leads to particles with excellent mechanical properties.

A new method to produce lens-shaped hydrogels based on this material at very gentle conditions, room temperature, and short time has been developed ***(1)***.

A LentiKat (*see* **Fig. 1**) combines the advantages of small and large particles: minimized diffusion limitation caused by the thin structure is linked to easy separation because of the large diameter of the biocatalyst. It can be retained easily in the bioreactor (e.g., by a sieve).

In contrast to biopolymers PVA hydrogels are hardly biodegradable and show an excellent mechanical stability. A large elongation at break (350–450%) and even in long-time fermentations no significant abrasion could be observed.

Correctly stabilized LentiKats tolerate a maximum temperature from 50 to 55°C, and pH values between 3.1 and 8.5 were tested for several days or weeks without any signs of disintegration of LentiKats. The suitability for even sensitive biocatalysts is given by the gentle entrapment conditions—only a short time is needed at room temperature.

The inner pore structure of the matrix can be adapted by additives according to the needs of the cell or enzyme.

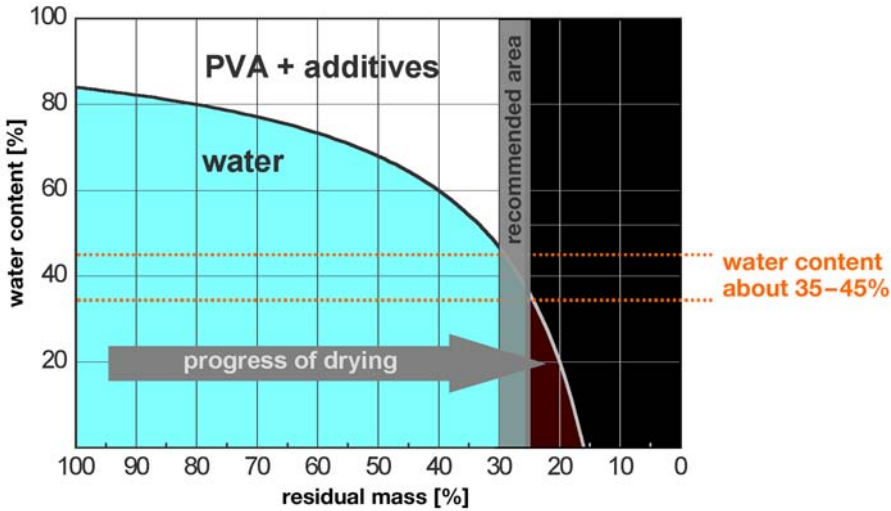

Fig. 2. Gelation by partial drying.

The principle of gelation is a partial drying of the biocatalyst-loaded polymer solution. The polymer solution (LentiKat®Liquid) is mixed with the biocatalyst, droplets have to be formed and dried to a certain water content (*see* **Fig. 2**). After a re-swelling step the LentiKats are ready to use.

2. Materials

1. LentiKat®Printer (GeniaLab, Braunschweig, Germany).
2. LentiKat®Liquid (GeniaLab; *see* **Note 1**).
3. LentiKat®Stabilizer (GeniaLab).
4. 145 mm Petri-dish ∅ = 145 mm (Greiner).
5. Sodium chloride (Merck, Darmstadt, Germany).
6. Carbol-Fuchsin solution (Merck).

3. Methods

3.1. Preparation of the Polymer-Biocatalyst Solution

The LentiKat®Liquid (*see* **Notes 1** and **2**) has to be melted by placing in a water-bath between 85 and 90°C. Use a magnetic stirrer for thorough mixing. Melt the polymer solution until the complete content of the bottle is a homogenous and clear liquid. The solution has to be cooled down to a temperature that is adequate for the biocatalyst directly before the immobilization process to avoid an undesired gelation process that will occur at lower temperatures. However, at a temperature between 25 and 30°C LentiKat®Liquid will be workable for several minutes.

After cooling, the biocatalyst solution is added and dispersed homogenously by mixing with a magnetic stirrer. In the case of immobilizing biomass wet matter (e.g., cells separated by centrifugation) the biomass has to be diluted by at least 50% with 1% sodium chloride solution before adding to LentiKat®Liquid.

Fig. 3. LentiKat®Printer, a tool to make uniform LentiKats® in lab-scale.

3.2. Production of LentiKats

For production of the LentiKats a smooth plate is needed, preferably of polystyrene. For example, single-use Petri dishes give good results. Some materials (e.g., glass) are not suitable at all. Each plate has to be marked individually to be weighed and the tare noted (*see* **Note 3**).

3.2.1. Using a Syringe

Using a standard syringe with a cannula (~1.0 mm in diameter), droplets need to be formed and dripped neatly and rapidly onto the surface of the plate (*see* **Note 4**). The droplets should be approx 3 mm in diameter, weigh 5 mg, and be as uniform as possible. After finishing a complete plate the amount of LentiKat®Liquid on this plate can be determined by balancing the whole plate again.

3.2.2. Using a LentiKat®Printer

For reproducible experiments in shaker flasks or small fermenters uniform particles are needed (*see* **Notes 5–8**). The easiest way to produce small amounts of immobilized catalyst is to use a special tool: the LentiKat®Printer (*see* **Fig. 3**). In one step, more than 400 uniform particles are formed and deposed on a Petri-dish. For a detailed description *see* "tips and tricks" in **ref. *3***. Up to 50 g of LentiKats can be produced easily in lab-scale, even under sterile conditions.

A smaller printer head is available with only 120 pins for smaller batches and reduced losses of material. This type is recommended when entrapping enzymes or other valuable materials.

3.3. Gelation and Stabilizing

After calculating the mass of the droplets the gelation by partial drying has to be launched. Drying can be done by leaving the plate simply exposed to air but to

accelerate the evaporation of water a ventilator or fan for aeration from above is useful. Maximum temperature during gelation should not rise above 35°C to avoid harm for the biocatalyst.

Drying of LentiKats is complete if 72% of the mass of LentiKat®Liquid that has been dripped on the plate has evaporated. For this reason it is advisable to control the weight of the plate with LentiKats regularly during gelation. The time needed for gelation strongly depends on the air draught and its temperature and humidity.

When gelation is complete the previously prepared LentiKat®Stabilizer is poured on the LentiKats for re-swelling and for sloughing the gel particles. LentiKats will reswell to about their original size by this treatment. After 3 to 8 min of contact with the stabilizer solution LentiKats are easily removed from the surface and put into a bottle containing a 10-fold surplus of LentiKat®Stabilizer. LentiKats have to be stirred for at least 2 h in stabilizer solution to give a good mechanical stability for the later application. The influence on LentiKat stability of any shortening in the times of operation given above have to be proved for each case.

After stabilizing is finished the supernatant has to be removed and replaced by the medium or solution with which you want to use your immobilized biocatalyst. If foaming occurs it may be necessary to remove and replace supernatant again after a certain time of stirring.

Some specific examples of the use of this system may be found in **Notes 9–12**.

3.4. Scale-Up of LentiKat-Production

For an industrial application using LentiKats a scale-up of the LentiKat production is necessary. We built up a pilot-plant for a continuous production in our institute (*see* **Figs. 4** and **5**). We chose a belt dryer using pretreated and dried air. A multinozzle system puts rows of 23 droplets on the belt.

After controlled drying in a 15-m tunnel the droplets are passed to a re-swelling area. A wiper takes the lenses off the belt and they fall into a stabilizing bath for hardening. Temperature and humidity are measured and controlled at different points in the drying tunnel and can be adjusted towards the desired water content of the droplets.

4. Notes

1. Commercialized LentiKat®Liquid is shipped sterile in a ready-to-use form. In the bottle are 80 g of the PVA-solution with various additives designed to mix with 20 mL of the biocatalyst solution resulting in 100 mL of LentiKats.
2. LentiKats are not only suitable for the immobilization of whole cells but also for enzymes ***(2)***.
3. These aspects should be taken into account before starting to immobilize:
   a. How much LentiKats should be prepared? Can you use a syringe or do you need a LentiKat®Printer?
   b. Are enough (sterile) Petri dishes and LentiKat®Stabilizer available?
   c. Do you want to use the LentiKats directly after the production or do you want to store them? Do you have you enough media? Does storing harm the entrapped biocatalyst?
4. Microorganisms entrapped in LentiKats can be controlled microscopically. To enhance contrast of cells and hydrogel, staining may be useful: treatment with

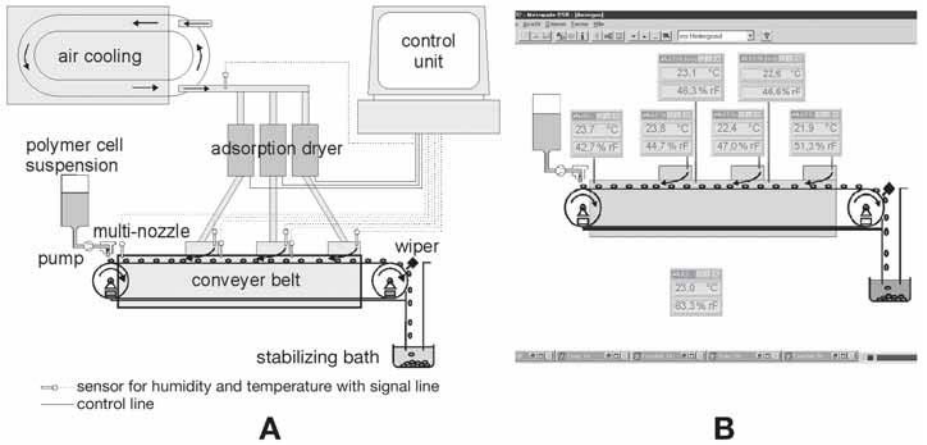

Fig. 4. **(A)** Sketch of the pilot-plant for continuous production of LentiKats. **(B)** Screenshot showing the actual values for humidity and temperature.

Fig. 5. Pilot-plant for continuous production of LentiKats at the Institute of Technology and Biosystems Engineering, Federal Agricultural Research Center, Braunschweig, Germany. **(A)** General view. **(B)** The multinozzle system puts rows of droplets onto the belt.

1:100 diluted Carbol-Fuchsin solution (ZIEHL and NEELSEN) gave good results after staining and decolorising of 30 min each.

5. If you are working with your biocatalyst entrapped in LentiKats you probably do not have to be as attentive to sterile conditions. Contaminating cells will not be able to enter the hydrogel and replace the wanted biocatalyst which hence will be in great surplus compared to any contaminating cells.

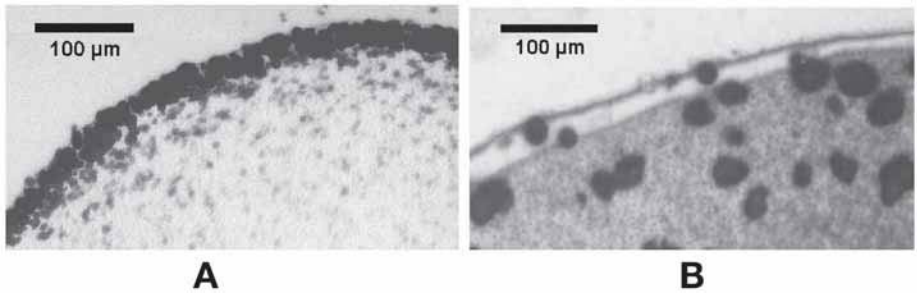

Fig. 6. Stained colonies at the edge of sliced LentiKats. **(A)** Too high initial biomass content. **(B)** Too low initial biomass content.

6. *Initial biomass of living cells.* If you are doing entrapment of living cells that should form colonies inside the LentiKats after immobilization the initial biomass content will without any doubt strongly influence the maximum specific activity you will find after growing to a steady state.
   - If initial biomass content is too high you will get shell-catalyst characterized by intensive biomass growth at the surface of LentiKats leading to very poor nutrient supply in the central part of the gel and bad specific activity (*see* **Fig. 6B**). If initial biomass content is too low the gel volume of LentiKats is not utilized sufficiently (*see* **Fig. 6A**). Hence, optimum for initial biomass content has to be determined for each application. As a rule of thumb, an initial biomass content of about 10^7 cells/mL of gel should be near optimum. This value was found for different bacterial strains. The cell number is more useful than weight of biomass since cells of different strains may strongly differ in their size, but one colony will have its origin in one cell not in a certain small portion of biomass.
   - If biomass content of LentiKats is too high some rolling of LentiKats may occur after some prolonged time of incubation. Besides decreasing biomass content there may be some relief by enlarging thickness of LentiKats or enhancing hydrogel stability (duration of stabilization or further drying if possible).
   - If dead or resting cells are to be entrapped a biomass content of 10% (w/w) biomass wet matter is recommended. If intracellular enzymes in more or less perforated cells ought to be used cross-linking the cell contents with glutardialdehyde might be helpful to prevent leakage of the biocatalyst.
7. The survival rate of microorganisms during immobilization can be determined by immobilizing a very small number of cells (e.g., 10^4 CFU/mL) and count the emerging colonies after staining. Subject to the assupmtion that the density of the biocatalyst polymer suspension and the LentiKats are about 1 kg/L and the hydrogel is re-swelling to its primal volume the theoretical number of colonies per LentiKat can be calculated and set to 100%.
8. The immobilization of the bacterium *Zymomonas mobilis* in LentiKats is described in detail in the following steps:
   a. To determine the optimal initial biomass content for this bacterium in LentiKats six batches with different initial biomass content were produced and their activity measured after they had grown to a steady state.

Table 1
Numbers of CFUs/mL Cell Suspension

Batch number	A CFU/mL (added to polymer)	B CFU/mL (in polymer/cell solution)	C Survived CFU/mL (= colonies/mL cat)
1	2.6×10^8	5.2×10^7	1.6×10^7
2	7.7×10^7	1.5×10^7	4.8×10^6
3	1.9×10^7	3.9×10^6	1.2×10^6
4	5.3×10^7	1.1×10^6	3.3×10^5
5	8.5×10^5	1.7×10^5	5.3×10^4
6	2.6×10^4	5.2×10^3	1.6×10^3

Note: Adding 20 mL of column A to 80 mL polymer solution leads to a fifth part of cells in column B. Assuming a survival rate for all batches of 31% leads to the number of colonies/mL LentiKat (column C).

b. Six 100-mL bottles containing 80 g of LentiKat®Liquid were heated in a waterbath until the polymer solution was completely clear and put in a drying oven at 60°C. Additionally, 1 L of LentiKat®Stabilizer was prepared.
c. Cells from the preparatory culture shortly before the end of the exponential period of growth were taken and the number of colony forming units (CFU) was counted using a hemacytometer. Twenty milliliters were added for each batch. Batch 1 was undiluted, batches 2 through 6 were diluted by media as shown in **Table 1**. The resulting number of CFU is also given **Table 1** and the survival rate is taken into account.
d. The survival rate is determined by counting the number of stained colonies from at least 20 LentiKats from batch 6 and generating the average value. Then the theoretical number of colonies for a LentiKat of this batch was calculated. From each batch, one Petri dish was printed with 413 LentiKats by the LentiKat®Printer so that 2.55 g of the polymer cell suspension is on each dish. At a density of 1 g/mL, and the assumption that the catalysts re-swell to their original size, 1, LentiKat has the volume of 6.17 µL and a mass of 6.17 µg, respectively.
e. The printed Petri dishes were dried under an exhaust hood with an extra fan installed 50 cm above the dishes. The LentiKats were dried to a residual mass of 28% (in this case when the droplets on one Petri dish weigh 0.714 g from the initial 2.55 g). This took about 40 min but this time is strongly dependent on humidity, temperature, air draught, and the size of the droplets. Times can vary from 20 to 80 min. The previously prepared LentiKat®Stabilizer is then poured on the LentiKats for re-swelling and for sloughing the gel particles. The LentiKats were transferred into an anaerobic workbench and put into media.
f. The theoretical number of colonies per LentiKat for batch 6 is, in this case, 32 (the observed average was only 10). This is equivalent to a survival rate of 31% which is assumed for the other batches as well because they were all treated in the same way (e.g., contact time to the polymer, drying time, contact time to oxygen, temperatures).
g. All batches were incubated anaerobically and stirred at 30°C at a start pH of 5.0 and a glucose concentration of 150 g/L. The media was changed twice a day. The

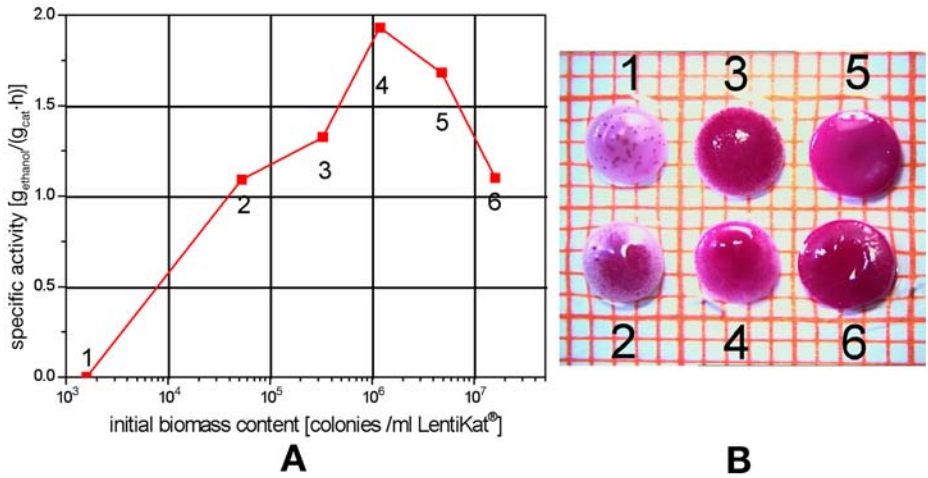

Fig. 7. Six batches with different initial biomass content (*see* **Table 1,** column *C*). **(A)** Specific activity of the batches after reaching a steady-state of activity. **(B)** Stained LentiKats from the different batch.

specific activity of the 6 batches was determined after 1, 4, and 6 d and found stable after 4 d. For the activity test each batch was incubated for 2 h and washed threefold with anaerobic media at constant temperature (30°C) before starting the real activity test in a 20-fold excess of media. The ethanol concentration is measured at the beginning and then every hour until the supernatant of the batches becomes turbid

h. In this case the optimal initial biomass is 1.2×10^6 (*see* **Fig. 7**), which is obviously lower than the rough rule of thumb given before.

i. To produce highly active LentiKats with *Zymomonas mobilis* the initial biomass content should be near to this optimum. Keep in mind that the survival rate within immobilization can vary strongly with the state of growth and should be controlled for each immobilization batch.

9. *Bioconversion of raw glycerol to 1,3-propanediol by* Clostridium butyricum *immobilized in LentiKats* ***(4)***. A growing interest in 1,3-propanediol as a new commodity chemical has been observed over revent years. 1,3-Propanediol has a great potential as a new compound for the production of polymers with excellent chemical and mechanical properties.

   There exists an interesting alternative to synthesis of 1,3-propanediol based on crude oil because various bacterial strains among the *Clostridium butyricum* are able to convert glycerol to 1,3-propanediol under oxygen exclusion with good yield.

   By entrapment of *C. butyricum* in LentiKats the efficiency of biotechnical 1,3-propanediol production can be improved. This hydrogel entrapment offers the opportunity to run the process under nonsterile conditions over several weeks without any risk of contaminating microorganisms. Thus, entrapment into the gel lenses leads to a simple but stable process.

With immobilized cells of *C. butyricum*, a productivity of more than 30 g 1,3-propanediol per liter per hour was achieved in continuous fermentation thatt is 5- to 10-fold higher as compared with conventional fermentation. Raw glycerols from biodiesel production wre utilized for bioconversion to 1,3-propanediol without any loss in activity.

10. *Significant reduction of energy consumption for sewage treatment by using LentiKat-encapsulated nitrifying bacteria* ***(5,6)***. Bacteria from an external fermentation were employed in entrapped form to specifically increase the nitrification rate in nitrifying waste-water treatment plants. For maximum biological and mechanical stability, LentiKats were chosen as immobilization support. Wastewater from a municipal wastewater treatment plant was used directly for the lab-scale setup and the parameters ammonia, nitrate, and chemical oxygen demand (COD) were measured regularly. The setup was run with different parameters over a period of 650 d. A specific volumetric nitrate production rate of approx 25–30 mg/(L·h) was achieved at maximum nitrification. The residence time was between 30 and 60 min, which is 10 times shorter as compared with conventional methods. For real wastewater treatment plants this means a significant reduction in size for the nitrification reactors. The COD consumption showed values between 10 and 50% because of specific nitrification. The remaining COD is available for the subsequent denitrification step. The LentiKats showed no deterioration over the complete time span.
11. *Bioethanol production from nonsterile molasses by yeasts*. Bioethanol is one of the most suitable energy carriers to reach the goal of the European Union to increase the biofuel rate to 5.75% in 2010 for transport purposes.

    In a three-step pilot scale process with yeasts immobilized in LentiKats, a productivity of 18 $kg_{EtOH}/(m^3 \cdot h)$ could be obtained from nonsterile molasses without pH adjustment. The productivity of industrial production (batch, free cells) is in the range of 1.3–1.5 $kg_{EtOH}/(m^3 \cdot h)$. Based on these results a conventional process concept was compared with of a process concept with yeasts immobilized in LentiKats. Only three fermenters with 60 m^3 each were needed instead of six fermenters each with 200 m^3 to produce 2500 L/h bioethanol.
12. *Production of (R)-cyanohydrins by using entrapped (R)-oxynitrilase in LentiKats* ***(7)***. The (*R*)-oxynitrilase (hydroxynitrilase lyase, E.C. 4.1.2.10), which catalyzes the reversible condensation of hydrogen cyanide with aldehydes and ketones, is a useful and promising enzyme for biotransformations. The resulting optically active cyanohydrins are versatile synthetic intermediates to prepare several important classes of chiral compounds: α-hydroxy acids and their esters, β-amino alcohols, β-hydroxy-α-amino acids, α-hydroxy aldehydes, and α-hydroxy ketones. These compounds are important precursors for pharmaceuticals and agrochemicals.

    Because costs for the enzyme and the efforts for recovering the soluble biocatalyst from the reaction mixture are high, an industrial use is not economic yet. By entrapping the enzyme a repeated use is possible and thus efficiency of the process is raised. Of course the applied immobilization technique has major impact on technical feasibility: it has to be effective and cheap at the same time.

The activity of (*R*)-oxynitrilase was assayed by synthesis of (*R*)-mandelonitrile. The reaction was performed in a weak acidic buffer solution (pH 3.8) at a low temperature (20°C) to diminish the competing nonenzymatic formation of a racemic mixture. Excellent enantiomeric excess (>99% ee, determined by HPLC analysis) was obtained.

For industrial use of enzymes the long-term stability is a crucial economical criterion. Hence, the (*R*)-oxynitrilase was entrapped in hydrogels to study the efficiency and the long-term stability of this system. The entrapment of (*R*)-oxynitrilase in hydrogels was based on a very simple two-step procedure. First, the molecular weight of (*R*)-oxynitrilase (60,000 g/mol) was increased by cross-linking and then the cross-linked enzyme was entrapped in LentiKats. The activity of the cross-linked (*R*)-oxynitrilase was 65% of its native activity; after entrapping, 89% of this activity remained. The entrapped (*R*)-oxynitrilase was used repeatedly and the activity was stable for at least 25 batches.

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Encapsulation of Cells in Alginate Gels

Gorka Orive, Rosa María Hernández, Alicia Rodríguez Gascón, and José Luis Pedraz

Summary

Cell encapsulation represents one of the current leading methodologies aimed at the delivery of biological products to patients for the treatment of multiple diseases. Alginate is the most frequently employed material for the elaboration of the polymer matrix and outer biocompatible membrane because of its mild gelling and biocompatibility and biodegradability properties. However, its successful exploitation requires knowledge of microencapsulation technology and of the main properties of alginates, including their composition, purity, and viscosity. This chapter will focus on the immobilization of cells in alginate gels to create alginate-PLL-alginate microcapsules using an electrostatic droplet generator.

Key Words: Alginate; biocompatibility; biomaterial; cell encapsulation; microcapsules.

1. Introduction

Biomaterials are already having an enormous impact on medicine. Fabrication of pure and highly biocompatible polymers have allowed scientists to apply them in multiple scientific areas, including tissue engineering ***(1)***, dental implants, wound dressings ***(2)***, and drug delivery ***(3,4)***. In order to provide different alternatives for each specific approach, a large variety of natural and synthetic polymers are currently available in medicine. In particular, natural polymers are attractive biomaterials because of their abundance and apparent biocompatibility. One of the most frequently employed natural polymers is alginate. Because of its mild gelling and biocompatibility and biodegradability properties, alginate has long been used in the food and pharmaceutical industries ***(5)***. Interestingly, there has been an increasing interest in using alginate in a wide range of scientific fields such as oral vaccination ***(6)***, development of controlled-release systems for conventional drugs and peptides ***(7,8)***, and cell microencapsulation for the controlled and continuous delivery of therapeutic products ***(9)***.

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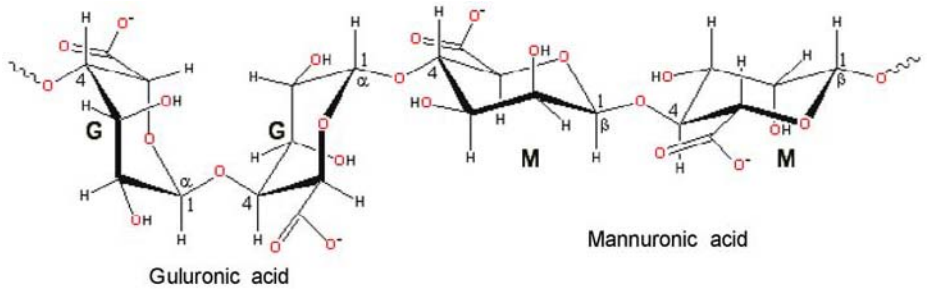

Fig. 1. Chemical structure of the alginates. G corresponds to guluronic acids, whereas M belongs to mannuronic blocks.

1.1. Alginates

Alginates are naturally occurring polysaccharides extracted from the brown seaweeds (*Phaeophycea*) found in the shallow waters of temperate zones. Although alginate was first prepared by Stanford and a pure isolation was afterwards achieved by Krefting ***(10)***, both in the 1880s, it was not until the 1930s that alginate was first exploited commercially. Different species of brown seaweeds, such as *Laminaria hyperborea* and *Macrocystis pyrifera*, are responsible for producing alginates with different chemical composition. In fact, as shown in **Fig. 1**, alginates are a family of unbranched binary copolymers of 164 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G). The alginate monomers occur as regions made up exclusively of one unit of M blocks or G blocks or as alternating structures (MG blocks) ***(11)***.

Because alginates do not have any regular repeating unit, the polymer chain cannot be described by Bernoullian statistics. Therefore, apart from the monomer composition, the diad and triad sequences must be measured by nuclear magnetic resonance (NMR) techniques if a complete characterization of the polymer is intended ***(12)***.

Numerous studies have shown that the monomeric composition, the molecular sequential structure, and gel-forming kinetics of an alginate have significant impact on some of its properties, including stability, mechanical resistance, permeability, biodegradability, and swelling behavior. Interestingly, there is a disagreement in the literature about the possible effects of all these properties on the biocompatibility and immunological characteristics of alginates and alginate microcapsules ***(13–15)***. Overall, it seems reasonable that purification of the polymers is a fundamental prerequisite for obtaining materials with improved biocompatibility properties. This can be observed clearly if nonpurified and purified alginates are tested using a tumor necrosis factor (TNF)-α production assay. As shown in **Fig. 2A**, the production of TNF-α (a proinflammatory cytokine involved in multiple inflammatory signals) ***(16)*** was approximately 100 times higher in a nonpurified alginate comparing with purified alginates of different composition and viscosity. Assuming this, the implantation of capsules elaborated with alginates of different degrees of purity gives as a result a completely different in vivo inflammatory response as shown in **Fig. 2B**. The presence of a fibroblast

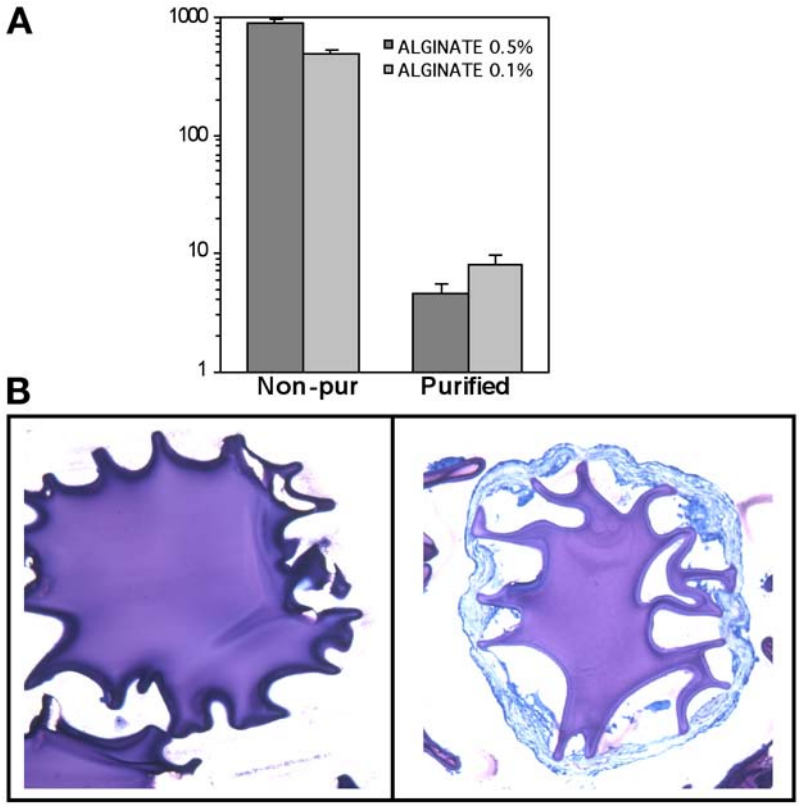

Fig. 2. (**A**) TNF-α secretion induced by different concentrations of a nonpurified and a purified low viscosity M-rich alginate (unpublished data). (**B**) Sections of alginate capsules recovered 1 mo after implantation. *Left:* nonfibrotic response; *right:* visible fibrotic response. Note the fibroblast overgrowth over the capsules (unpublished data).

growth layer over the capsules could impair effective secretion of the therapeutic protein from the immobilized cells, while also causing a metabolic obstacle to implantation by decreasing diffusion of oxygen, which then leads to graft failure.

1.2. Alginate Gels: Elaboration of Beads and Capsules

Under controlled conditions, alginates will form gels with a large number of divalent cations. The rigidity of the ionic alginate gels increases generally with the affinity of the ion in the order: Mn>Co>Zn>Cd>Ni>Cu>Pb>Ca>Sr>Ba ***(17)***. However, most of these ions cannot be used for immobilization of therapeutic active cells. In general, Ca^{+2} is the most frequently employed ion for such purposes because of its low toxicity. The gel-forming ability of the alginate is mainly related to its content of guluronic acid. In fact, diaxially linked G blocks create a cavity that acts as a binding site for the cation (**Fig. 3**).

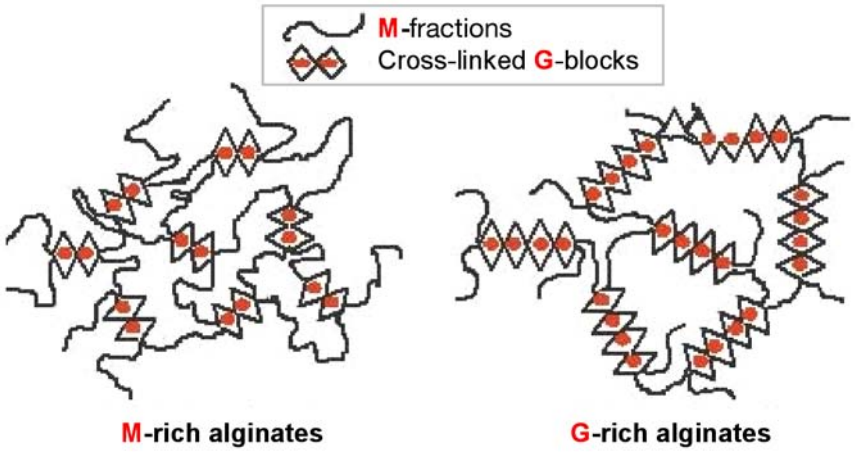

Fig. 3. Network structure in gels made from alginates with guluronic blocks of different lengths.

Gel strength depends on the molecular size and the composition of the alginates. For example, high-G alginates bind the divalent ions better than alginates with low content in guluronic blocks, making gels with in general highest mechanical strength ***(18)***, more stability to chelating compounds ***(19)***, and higher porosity. The latter can be explained because high-G alginates, with their long G-blocks and short elastic segments, create more open and static networks compared with low-G alginates. Other variables that must be taken into account when preparing alginate beads include: type and viscosity of the alginate, pH, temperature, presence of sequestrants in the cross-linking solution (such as EDTA or citrate), source, and concentration of calcium ions, and microencapsulation method.

One alternative for making more stable and less porous gels is to fabricate homogeneous core networks. Because alginate gels are usually formed by diffusing cross-linking ions into an alginate solution, gels with varying degrees of anisotropy with respect to polymer concentration can be formed by controlling the kinetics of the gel formation ***(20)***. Simply by addition of nongelling ions (such as sodium or potassium) to the cross-linking solution, the coupled diffusion between alginate and counter ions is impaired and more homogeneous gels are formed. Another way to improve the stability and reduce the porosity of alginate beads was proposed 25 yr ago by Lim and Sun and it consisted of coating the cell-loaded cross-linked beads with a polycation such as poly-L-lysine (PLL) ***(21)***. By simply suspending the the negatively charged alginate droplets in the positively charged PLL solution, a detectable and mechanically resistant semipermeable membrane will be formed around the beads in 5–10 min. In a subsequent step, capsules are coated again with an alginate layer to improve the biocompatibility of the system. This system will be explained in detail later. The pioneering work of Lim and Sun opened the door to innumerable novel cell encapsulation protocols in which the elaboration conditions, the cells, the polycations, or all of them were changed to

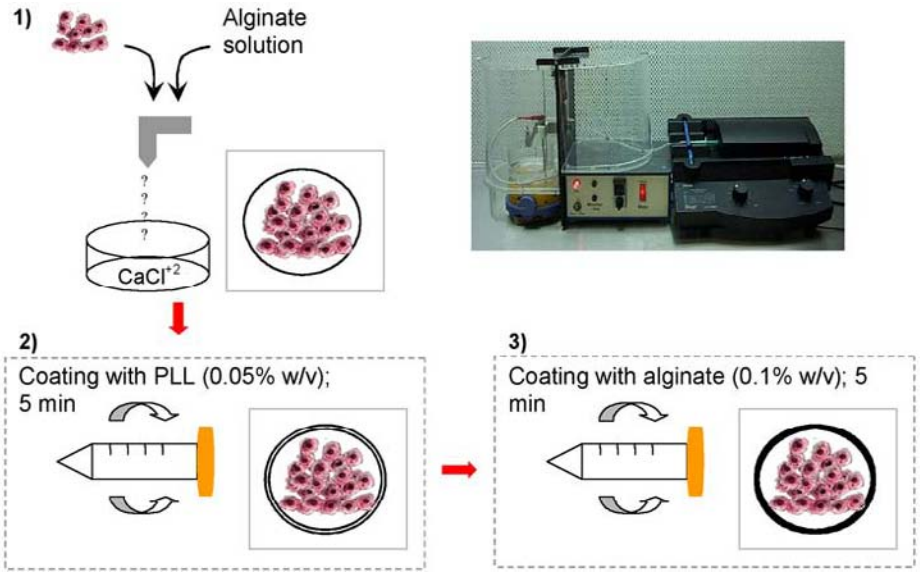

Fig. 4. Elaboration of cell-loaded alginate–PLL–alginate microcapsules using an electrostatic droplet generator. Initially, cells and alginate solution are mixed and the suspension is extruded using the electrostatic device. Because only $CaCl^{+2}$ is used as cross-linking solution, inhomogeneous core beads with improved mechanical stability will be formed. The addition of NaCl to the collecting solution will result in homogeneous core beads (**1**). In a second step, beads are washed and coated with a 0.05% (w/v) PLL solution for 5 min (**2**) and finally the resulting capsules are incubated with a 0.1% (w/v) alginate solution to create the outer biocompatible layer (**3**).

improve some key properties such as permeability, biosecurity, reproducibility, biocompatibility, and mechanical stability ***(22–25)***.

Another focus of interest is the selection of an appropriate microencapsulation method. In general, elaboration of cell-loaded alginate–PLL–alginate capsules involves two steps: droplet formation and gelation of the droplets in a cross-linking solution. The former can be realized using different techniques, such as the application of a coaxial air jet or a liquid flow to increase the force acting on nascent drops, the use of an electrostatic potential between the capillary and the collecting solution to improve drop formation while reducing size distribution, or the employment of a vibrating or a rotating capillary jet breakage to break the alginate–cell suspension jet into small droplets.

In this chapter, we will focus on the immobilization of cells in alginate gels to create alginate–PLL–alginate microcapsules using an electrostatic droplet generator. The encapsulation system (**Fig. 4)** is basically composed of a droplet generator device and a peristaltic pump governaed by a number of variables that need to be optimized for optimal microcapsule elaboration. The effect of such variables on the size of the resulting beads will be discussed later.

1.3. Immobilization of C_2C_{12} Cells in Alginate–PLL–Alginate Microcapsules

C_2C_{12} myoblasts have been selected as model cells for their immobilization because they have been broadly employed in literature as somatic gene therapy approach using cell encapsulation technology. Furthermore, C_2C_{12} cells can differentiate terminally into myotubes both in vitro ***(26)*** and after immobilization ***(27)***. In this way, they could reach a numerically stable population set by the carrying capacity of the microcapsule, which is an advantage to redefine the dosage for in vivo application. On the other hand, the microcapsules proposed here are made of a calcium–alginate matrix and PLL–alginate semipermeable membrane.

2. Materials

2.1. General Reagents

1. Sodium alginate (FMC Biopolymer, Norway *or* Sigma, St. Louis, MO; *see* **Note 1**).
2. Poly-L-lysine (PLL) hydrochloride (mol wt: 15,000–30,000; Sigma).
3. Calcium chloride solution, mannitol, sodium chloride (Sigma).
4. Hanks solution (*see* **Note 2**).

2.2. Cell and Culture Mediums

1. C_2C_{12} myoblast cells (from LGC/ATCC batch number CRL-1772).
2. Culture mediums: DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution for the myoblast cells. To differentiate the myoblast into myotubes the complete medium was supplemented with 2% horse serum instead of FBS. Reagents from Gibco-BRL (Invitrogen S.A., Spain).
3. Cell passes every 2 d. Cell cultured at 37°C in a humidified 5% CO_2/95% air atmosphere.
4. The flasks, pipets, centrifuge tubes, and the remaining materials must be sterile.

2.3. Electrostatic Droplet Generator

The following parameters must be optimized in order to fabricate uniform and reproducible microcapsules using an electrostatic device: needle diameter, electrostatic voltage, flow-rate, and distance between the needle and the gelling solution. The influence of each parameter on the size of the droplets is reviewed in **Table 1**. The size of the capsule is critical because it has direct effect on mass transfer of nutrients and especially of oxygen, a key parameter for cell survival. Some studies show that anoxia can occur in the center of spheres of 250 μm in diameter ***(28)***. Smaller microcapsules have been shown to present better biocompatibility ***(29,30)*** than larger ones but, on the contrary, they show lower stability ***(31)***. Experts in the field have considered 400–450 μm as an optimal diameter for cell immobilization. Using an electrostatic device it is possible to obtain capsules of 250 μm in diameter with aceptable size dispersions.

3. Methods

3.1. Preparation of the Cell Pellet (see Note 3)

1. Wash the cells attached to flasks several times with PBS solution, pH 7.4.

Table 1
Effect of the Main parameters Affecting Microcapsules Production by Electrostatic Droplet Generator (unpublished data)

↑ Needle diameter	↑ Diameter of the droplets
↑ Electrostatic voltage	↓ Diameter of the droplets
↑ Flow rate	No significant changes
↑ Distance from needle to solution	↑ Diameter of the droplets

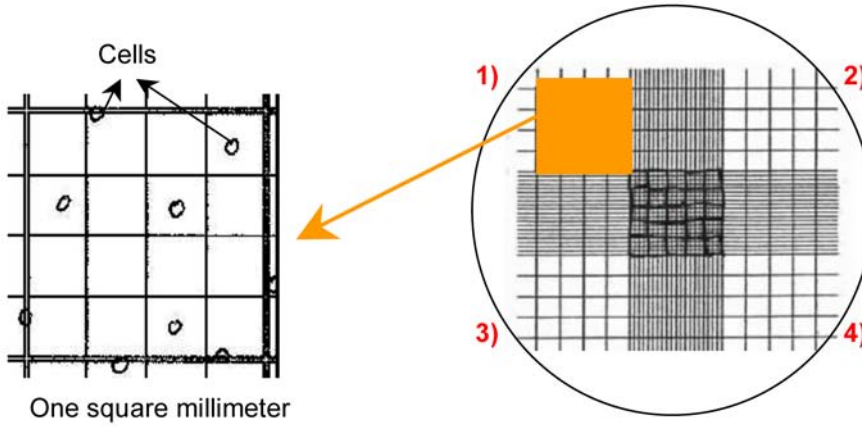

Fig. 5. The hemocytometer is a simple method for the enumeration of eukaryotic and prokaryotic cells. It is made of a glass slide that is viewed under a light microscope that consists of two identical counting chambers with a series of grids and a fragile (expensive) coverslip. Under the microscope it is possible to see nine fields that are each 1 mm square. However, only cells deposited in the four peripheric fields will be counted (*right*). On the left, an extended vision of one of the field in which the the circles represent cells suspended in the chamber between the slide and the cover slip.

2. After washing, add between 5 and 10 mL of trypsin–ethylene diamine tetraacetic acid (EDTA) (Gibco-BRL) to the flasks to provoke the detachment of the cells.
3. Once cells are detached, add 3 mL of complete culture medium to the flasks to stop the effect of the trypsin–EDTA solution.
4. At this point, transfer the cell suspension to a 50 mL centrifuge tube and determine the cell density using an hemocytometer as shown in **Fig. 5**.
5. Centrifuge the cell suspension to obtain a pellet.
6. Redisperse the cell pellet in a known volume of alginate to obtain the desired final cell density.

3.2. Preparation of the Reagents (see Note 4)

1. Prepare a 1.5% (w/v) sodium alginate solution (LVG: low viscosity alginate rich in gulutonic blocks) in Milli-Q water with 1% (w/v) mannitol. Ten milliliters of

the alginate solution is enough for three batches of 2-mL of capsules. This solution will be extruded to form the alginate droplets
2. Prepare a 0.05% (w/v) PLL solution in Milli-Q water with 0.9% (w/v) NaCl. Twenty milliliters of this solution are needed to coat 2 mL of alginate droplets.
3. Prepare a 0.1% (w/v) sodium alginate solution in Milli-Q water with 1% (w/v) mannitol. Twenty milliliters of this solution are needed to create a outer layer in 2 mL of alginate droplets.
4. Prepare a 55-m*M* calcium chloride solution in Milli-Q water with 1% (w/v) mannitol. Eighty milliliters of this solution are needed per capsule batch.
5. Sterilize all the solutions with a 0.22-μm filtration unit.

3.3. Elaboration of Cell-Loaded Microcapsules (see Note 5)

1. Mix the cell pellet with the corresponding volume of alginate solution to obtain the desired cell density.
2. Transfer the alginate–cell suspension to a syringe and connect the latter to the peristaltic pump.
3. Connect the syringe to a 0.4-mm needle using a 22-cm-length plastic tube. In this way, the alginate–cell suspension will be transferred from the peristaltic pump to the electrostatic generator
4. Incorporate the cross-linking solution inside the electrostatic device (*see* **Note 6**).
5. Switch on the peristaltic pump and the electrostatic device. Variables: needle diameter 0.35 mm, voltage 8 kV, flow-rate 5.9 mL/h, and distance from needle to gelling solution 25 mm (*see* **Note 7**). These variables enable the elaboration of microcapsules with a mean diameter of 450 μm.
6. Extrude the alginate–cell suspension until the desired bead number is obtained.
7. Stop the electrostatic droplet generator and the peristaltic pump. Be aware that there is no alginate–cell suspension in the needle because without electrostatic potential big droplets will be formed.
8. Leave the cell-loaded alginate beads in the calcium chloride solution for 5–10 min to induce the total polymerization of the alginate.
9. Filter the bead solution to eliminate the satellite peaks (*see* **Note 8**).
10. Wash the filtered beads with Hanks' solution.
11. Tranfer the beads to a 50-mL centrifuge tube and incubate them with a 0.05% (w/v) PLL solution for 5 min (*see* **Note 9**).
12. Filter the cell-loaded capsules to eliminate the nonbound PLL.
13. Wash the capsules again with Hanks' solution.
14. Tranfer the beads to another 50-mL centrifuge tube and incubate them with a 0.1% (w/v) alginate solution for 5 min.
15. Wash and filter the capsules and culture them in complete culture medium.
16. Additionally, it is possible to prepare liquefied capsules by incubating the solid spheres in citrate solution (55 m*M* for 5 min) as shown in **Fig. 6** ***(32–34)***.

4. Notes

1. Selection of alginates must take into account some variables, such as viscosity and monomeric acid composition. FMC Biopolymer, Norway, sells four differ-

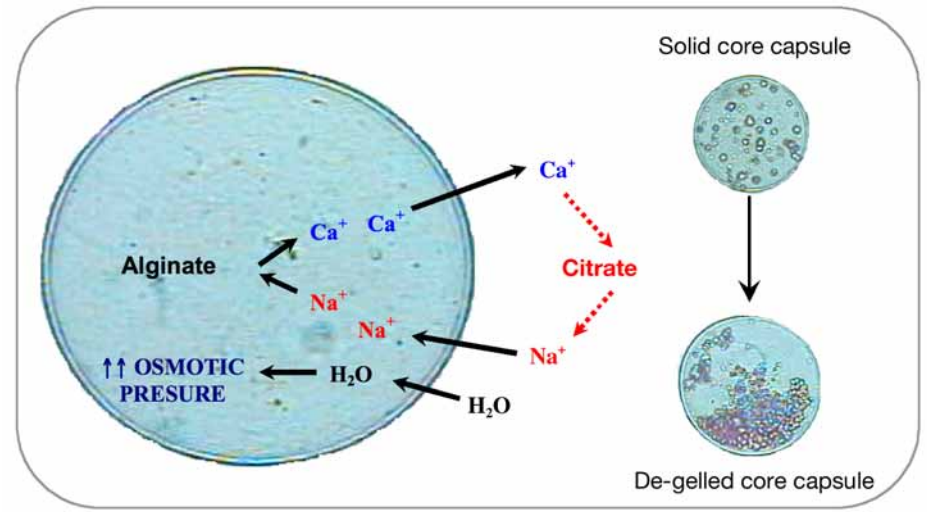

Fig 6. The treatment of solid core capsules with citrate provokes the de-gelling of the capsule by the exchange of calcium ions by sodium ions. This induces the entrance of water inside the capsule increasing the osmotic pressure. As a consequence an increase in the diameter of the capsule and the free movement of the cells within the matrix is obtained.

ent purified alginates: LVG, low-viscosity guluronic acid rich alginate; LVM, low-viscosity mannuronic acid rich alginate; MVG, medium-viscosity guluronic acid rich alginate; and MVM, medium-viscosity mannuronic acid rich alginate.

2. Hanks' solution, pH 7.4, is composed of 125 m*M* NaCl , 4.8 m*M* KCl, 1.3 m*M* $CaCl_2$, 25 m*M* HEPES, 1.2 m*M* Mg_2SO_4, 1.2 m*M* KH_2PO_4, 1 m*M* ascorbic acid, and 5.6 m*M* glucose.
3. Cells selected for immobilization need to be detached from the flasks and counted using a hemocytometer (Sigma; *see* **Fig. 5**).
4. Cell microencapsulation is a technological process that must be carried out in sterile conditions to avoid the risk of any contaminations of the immobilized cells.
5. It is convenient to adjust the pH of the solutions to 7.4
6. Nonhomogeneous microcapsules are prepared using calcium chloride as cross-linking solution while homogeneous ones need the addition of sodium chloride.
7. Check that the extrusion process is correct and that suitable and uniform droplets are being fabricated.
8. Satellites are formed by breakage of the fine filament between the droplet and the needle tip just before separation, resulting in secondary peaks.
9. The concentration and incubation time of the polycation is optional. The increase of both the concentration and the coating time will provoke a reduction in the size of the capsules and a thicker membrane with a reduced permeability properties.

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Immobilization of Cells on Polyurethane Foam

Ignacio de Ory, Gema Cabrera, Martin Ramirez, and Ana Blandino

Summary

In this chapter, protocols and details for the immobilization of a model cell onto polyurethane foam carriers are provided in order to facilitate the use of such systems in laboratory or industrial reactors. Polyurethane foam has recently acquired great relevance as a carrier for its good mechanical properties, high porosity, and large adsorption surface. In addition, it has a very low commercial cost. Two different immobilization protocols have been described, differing in the flow regime or the possibilities for the reactor: immobilization in a stirred tank reactor working in a discontinuous regime (by cycles) and immobilization in a packed column working in continuous operation mode. Protocols for carrier sterilization, analytical methodology, and immobilization are described.

Key Words: Adsorption; packed column; polyurethane foam; stirred tank.

1. Introduction

Adsorption onto surfaces represents a particular form of cellular adhesion based on the ability of certain microorganisms to fix themselves to solid surfaces by means of natural physicochemical bonds ***(1)***. Polyurethane foam (PUF) has recently acquired great relevance as a carrier in this immobilization technique, a fact exemplified by the increasing number of applications reported in literature. These applications include removal of organic compounds ***(2–6)***, odor waste control ***(7)***, acetic acid fermentation ***(8)***, hydrocarbon removal ***(9)***, and ferrous sulfate oxidation ***(10)***. In such studies, several microorganisms were immobilized (or coimmobilized) onto this carrier using different types of reactors such as stirred tanks, packed columns or biofilters.

PUF is an inert material with good mechanical properties (high resistance and elasticity) and very low commercial cost. This material also has a high porosity (near 97%) and therefore has a large adsorption surface. In addition to this, PUF does not suffer from the scale-up drawbacks experienced with other encapsulation matrices because huge volumes of this carrier can be easily prepared. Another

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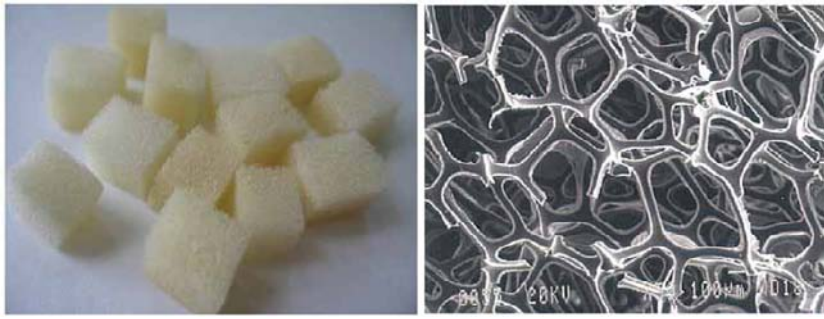

Fig. 1. Photograph of cubes of polyurethane foam and its internal structure (original magnification 50).

important advantage of this immobilization support is that oxygen diffusion problems can be reduced as a result of its large pore size, which is a particularly relevant factor for aerobic microorganisms. Cubic units of commercial PUF and an electron micrograph of its internal structure are shown in **Fig. 1**.

One aspect that must be pointed out in order to understand the phenomena involved in the immobilization process on PUF is the adsorption–desorption equilibrium in the biofilm. When the immobilized biofilm is forming (adsorption) a continuous loss of cells is simultaneously occurring (desorption), especially if the reactor is energetically stirred. For this reason, a suitable stirring rate must be established to find a satisfactory balance between the two effects. On the one hand, vigorous stirring improves the general conditions for mass transfer to submerged biomass and, as a consequence, increases the total biomass population and the number of cells adsorbed onto the carrier. However, both erosion effects and collision tensions can be increased by turbulent stirring, seriously hindering surface cell adsorption processes ***(11,12)***. At the same time, the amount of interstitial biomass (cells submerged in the liquid remaining within the internal structure of PUF) constitutes a crucial source of potential immobilized biomass for the continuous adsorption–desorption equilibrium and, in its own right, has great relevance for the success of subsequent fermentations ***(13)***.

In this chapter, protocols and details for the immobilization of a model cell onto PUF carriers will be provided in order to facilitate the use of such systems in laboratory or industrial reactors. Not all cells have the ability to fix themselves to solid surfaces, but they usually take advantage of the symbiotic action of other microorganisms in a mixed culture. These other microorganisms are adhered but do not contribute to the biological reaction. A number of studies concerning immobilization with mixed cultures or co-immobilization are reported in the literature ***(14)***. In general, all bacteria have this single adsorption capacity and so, in order to develop a more generic model, aerobic bacteria will be considered.

Two different immobilization protocols will be discussed and these differ in the flow regime or the possibilities for the reactor: (1) immobilization in a stirred tank reactor working in a discontinuous regime (by cycles) and (2) immobilization in a packed column working in continuous operation mode.

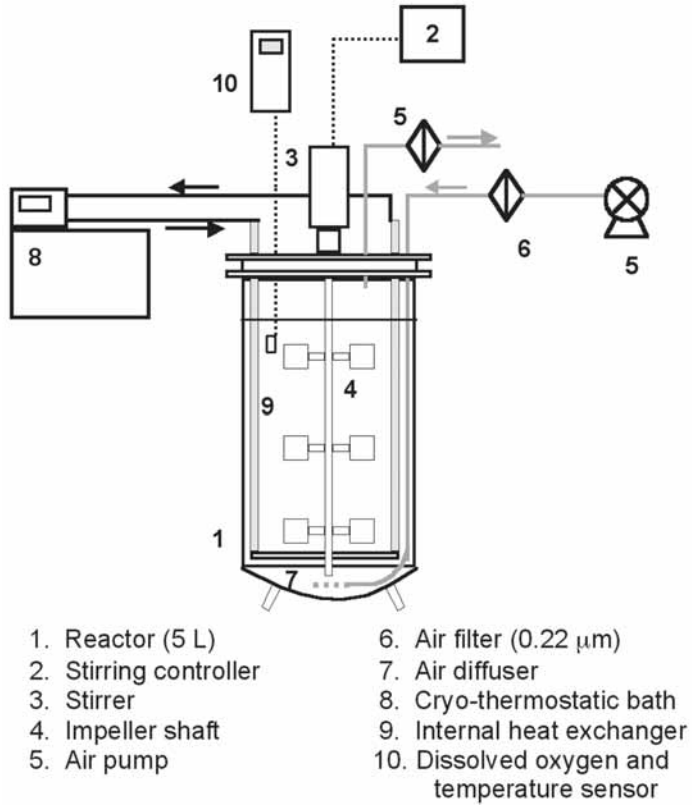

Fig. 2. Stirred tank reactor.

2. Materials

2.1. Reactors

1. Stirred tank reactor. A common cylinder-shaped vessel with a working volume of 5 L (total volume 7 L) is proposed. The reactor design allows a homogeneous distribution of liquid, avoiding dead volumes and decreasing the hydrodynamic stress. The system incorporates a stirring controller that allows a stirring rate between 0 and 500 rpm. Appropriate agitation is achieved with a set of stainless steel cross-blades. A thermostatic bath is connected to an internal heat exchanger in order to achieve the optimum temperature for the cells. An air pump, fitted to an amicrobic filter, performs the aeration for the reactor through a porous diffuser located underneath. The gas leaves through another air filter. This system is completed with a dissolved oxygen sensor and the reactor is filled with PUF cubes (20–25 g). The reactor is represented in **Fig. 2**.
2. Packed column reactor. A PVC tube (0.89-m height) with a total volume of 2.38 L is proposed. The working packed volume is 2 L (20 g of PUF cubes). Three sample ports are located along the column for the removal of solid units, and two

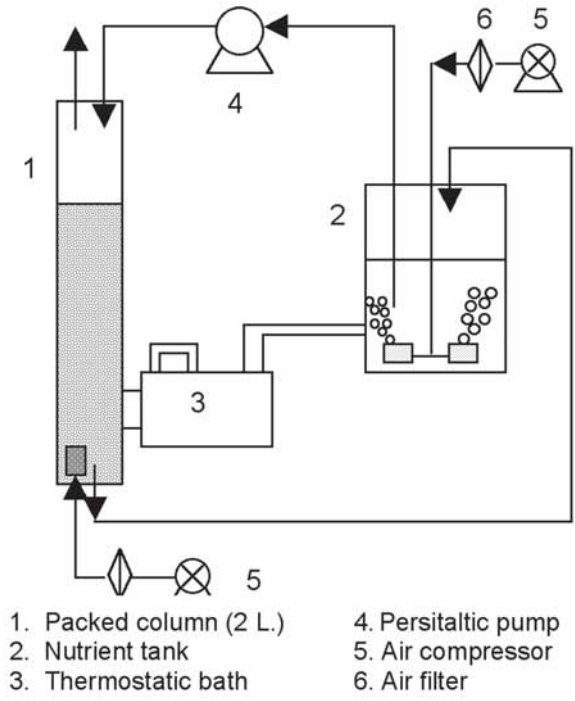

Fig. 3. Packed column reactor.

ports (input and output) for the sampling of liquid. The column is aerated from the base with an air diffuser that is connected to an air filter (0.22 μm pore size). The column is fed from the upper part with a peristaltic pump and the packed column is continuously submerged in the feeding liquid in order to avoid the formation of preferential routes and to obtain greater homogenization. The pump is linked with a nutrient tank (4 L), which contains an appropriate liquid medium for cell growth and is continuously aerated with an air compressor. The liquid effluent from the column feeds the nutrient tank. The column and the nutrient tank have a water jacket and are maintained at the optimum temperature. The system is represented in **Fig. 3**.

2.2. Preparation of Inoculum

Prior to the immobilization it is necessary to obtain a given quantity of free submerged cells to feed the reactors. Such an inoculum consists of a suitable fermentative medium with a selected strain of the cell in its exponential growth phase. This material provides the source of cells for the adsorption onto the foam. The appropriate literature for every microorganism provides particular protocols for the culture propagation, normally consisting of various scale-up steps. It is very important to point out that the activity of this culture is a decisive factor for the success of the subsequent immobilization.

2.3. Carrier Units and Sterilization

Cubic foam pieces (1-cm length) of commercial polyurethane are used. These units have a porosity of around 97% and an apparent density of 0.02 g/mL. The average pore size of the units is 400 μm (*see* **Fig. 1**).

Polyurethanes can be synthesized by the reactions of an alcohol with two or more hydroxyl groups and of isocyanates with more than one isocyanate group. This type of reaction is known as an addition polymerization. A combination of hydrogen bonding and the nature of the *R* groups gives rise to the range of properties observed for polyurethanes. A foam is formed when CO_2 gas becomes trapped within the polymer during the isocyanate reaction with water.

Both types of reactors are made from autoclavable materials and so they can be sterilized (120°C, 15 psi, 20 min) with the cubic foam units inside.

2.4. Analytical Methods

Different types of analysis have to be performed on the liquid medium and the carrier in order to follow the immobilization process.

1. Submerged biomass concentration in the liquid medium (viable and nonviable cells) is determined by optical microscopy using statistical re-count with a Neubauer chamber.
2. Substrate or product concentrations in the liquid medium must be analyzed with the aim of controlling the fermentation process.
3. Immobilized biomass concentration is measured using the following technique ***(8)***: a unit of carrier is removed from the reactor and squeezed lightly in order to remove the interstitial liquid. The carrier is then submerged in an Erlenmeyer flask containing 25 mL of a buffer solution with the optimum pH for the immobilized cell. The flask is placed in an ultrasonic bath at room temperature for 15 min. These conditions lead to the total desorption of adhered cells. In the final stage, the Neubauer chamber re-count method for the submerged cells is carried out on the liquid phase. The carrier is subsequently removed from the flask and dried in an oven at 80°C for 24 h. It is then possible to calculate the number of immobilized cell/mg of dry carrier . This technique must be validated prior to use by developing experiments concerned with cellular resistance to ultrasonic treatment.
4. Interstitial biomass concentration is calculated by removing the liquid inside the unit of carrier by light squeezing and determining the biomass occluded by means of optical microscopy using statistical re-count with a Neubauer chamber. As indicated above, this is an important reference to evaluate the success of the immobilization procedure.

3. Methods

The methodology of immobilization will be described for two different reactor configurations. The two systems described are the most commonly used in laboratory and industrial processes developed with the participation of fixed cells: stirred tank reactor working in a discontinuous regime (by cycles) and packed column working in continuous operation mode.

3.1. Discontinuous Stirred Tank Reactor

The general procedure involves carrying out consecutive fermentation cycles in a discontinuous operation mode, with the free microorganisms suspended in the liquid medium in the presence of the solid carrier (PUF cubes). Throughout the different cycles, the biomass gradually adheres to the carrier until a point of maximum adsorption capacity (biofilm saturation) is achieved. The experimental work is outlined in the following steps:

1. The 7-L stirred tank described in **Subheading 2.1.**, **item 1** is charged with 20 to 25 g of cubic units of PUF and the system is sterilized by wet heat in an autoclave.
2. 5 L of inoculum (growth medium with a large population of cells in exponential growth phase) is introduced into the reactor.
3. Optimum conditions for the growth of submerged cells are implemented in the reactor (e.g, aeration, stirring rate, temperature). The agitation causes the foam units to scatter throughout the liquid volume because of the low density of the foam. At this point, it is necessary to remember the importance of establishing a soft stirring rate, which guarantees appropriate levels of homogenization and oxygen transfer to the cell while avoiding the undesirable shear stress effects due to collisions between the units.
4. In the first cycle, the population of submerged cells will complete the consumption of the substrate and generate the product of the main biological reaction. The state of such cultures is followed by analysis of submerged biomass and evaluation of substrate and product concentrations.
5. When the cycle is complete, a sample of polyurethane foam (2 or 3 units) is removed from the reactor. A counting protocol for total immobilized biomass and interstitial biomass is then carried out. The next stage involves refreshing the liquid medium: a percentage of the total liquid volume is removed (approx 50%) and the reactor is then re-filled with the same volume of fresh growth medium (substrate). A new discontinuous cycle then starts.
6. Several discontinuous cycles are carried out (*see* scheme in **Fig. 4**). At the end of each cycle a sample of solid carrier is removed and analyzed. The concentration of immobilized cells progressively increases up to the maximum adsorption capacity. Normally, four to six cycles are sufficient (300–500 h). The immobilized cell concentration follows the trend shown in **Fig. 5**, which is explained in the **Note 1**.
7. Once the final saturation point has been reached, the carrier particles are ready to be used for fermentation purposes in the reactor or to be removed and used in another reactor.

3.2. Continuous Flow Through a Packed Column

This procedure involves circulating the culture medium through the aerated submerged column (from the top to the bottom), thus allowing the progressive fixation of the cells to the PUF cubes. The culture medium then flows back to the nutrient tank for a continuously recycling flow. The system can be summarized as follows:

1. The 2-L packed column described in **Subheading 2.1.**, **item 2** is filled with 20 g of cubic PUF units and the system is sterilized by wet heat in an autoclave.

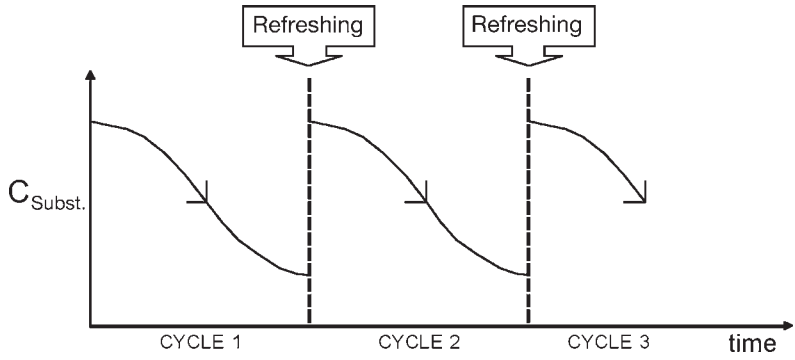

Fig. 4. Scheme showing discontinuous cycles carried out with the submerged culture in the stirred tank: Concentration of substrate vs time.

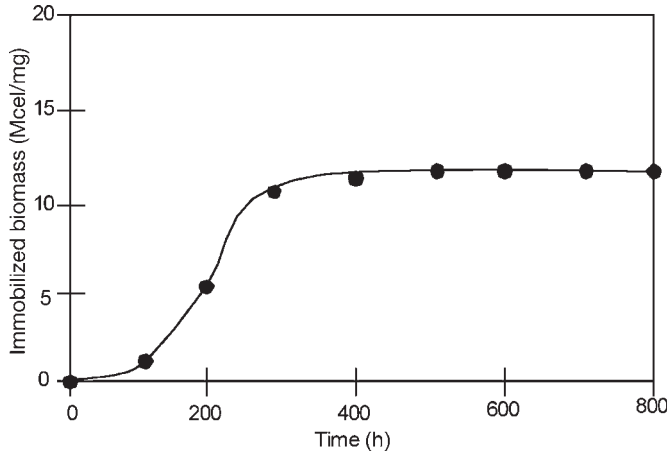

Fig. 5. Profile for immobilized biomass concentration (in millions of cell/mg of carrier vs time) in PUF cubes submerged in a discontinuous stirred tank.

2. The reactor is cooled and connected to the rest of the equipment. The 4 L of inoculum in the nutrient tank is allowed to begin to fill up the column from the top. An external pipe keeps the column continuously submerged and maintains a constant level of liquid. The effluent exiting from the bottom of the column returns to the nutrient tank.
3. A wide range of residence times (HRT) can be established in the reactor but, below a critical flow rate, this factor has little influence on the immobilization rate and the total adsorption capacity. A time of 1 h is recommended.
4. It is necessary to analyze the submerged biomass, substrate, and product concentrations in the input and output liquid on a daily basis. Furthermore, three samples

of PUF units (at different column ports) must be removed in order to estimate the immobilization degree of the carrier and the interstitial biomass. The nutrient tank must also be refreshed with new growth medium when the substrate has been exhausted.

5. A massive immobilization of cells will be registered almost immediately and will reach saturation on the carrier (steady state) in 200 to 300 h.
6. Once the final saturation point has been reached, the carrier particles are ready to be used for fermentation purposes in the reactor or to be removed and used in another reactor.

4. Notes

1. The trend followed by immobilized biomass, based on abundant experimental data, is shown in **Fig. 5**. This behavior can be explained in terms of the internal structure of PUF. After an initial stage of approx 100 h without any appreciable immobilization, a sudden increase in adhered biomass is observed (within the next 150–200 h). The maximum colonization of PUF is then reached. From this point on, no further adsorption in registered. This trend is directly related to the hydrodynamic behavior of the PUF submerged in the liquid phase. During the first hours of operation (discontinuous or continuous) the PUF cubes remain dry, but as the process continues they gradually become completely wet by capillary action. After that point, the adsorption begins at a high rate because of the highly porous structure of this carrier, which facilitates the total exposure of the surface and accesibility to the cells ***(8)***.
2. As indicated in the introduction, the stirring rate plays a very important role in determining the maximum adsorption capacity. Studies in the literature show that an increase in the stirring rate causes a decrease in the immobilization. As an example, the immobilization of *Acetobacter aceti* on PUF reaches 6 million cell/mg of carrier when the stirring rate is 200 rpm but increases to 13 million when the agitation rate is decreased to 125 rpm ***(13)***.

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32

Immobilization of Cells With Transition Metal

Pedro Fernandes

Summary

The use of transition metal-based supports for cell immobilization has proved its feasibility in some fermentative or bioconversion processes with potential for commercial-scale application in the food industry. It was therefore logical to evaluate the validity of this immobilization method within the development of other relevant bioprocesses, such as steroid bioconversions. Among the transition metal-based supports, titanium-based supports are by far the most widely used for whole cell immobilization. In order to illustrate this concept, some practical approaches for the immobilization of yeast and bacterial cells onto titanium-based supports, aiming for the production of biocatalysts with application in bioconversion processes, are presented in this work

Key Words: Immobilization; titanium; transition metals; whole cells.

1. Introduction

Among the multiple methodologies used for the immobilization of whole cells, an approach based on transition metal chemistry has proved feasible ***(1–5)***, particularly in processes related to the food industry, namely in wine-making and brewing ***(6–9)***. Other examples include bioconversion of sterols ***(5)*** and steroids ***(10)*** or vinegar production ***(11)***. This methodology is based on the nontoxicity of transition metal oxides toward microbial cells ***(12)***, and its application has also been extended to the immobilization of whole cells onto different inorganic or organic supports (e.g., celite, DEAE-cellulose) treated with a given transition metal. Although oxides of iron (III), vanadium (III), tin (IV), zirconium (IV), and titanium (IV) have been used for the immobilization of biomolecules ***(1,13,14)***, the latter is clearly the most commonly used transition metal for whole cell immobilization. Using a gelatinous hydrous metal oxide, cells can be immobilized onto transition metal supports. This approach involves the replacement of hydroxyl groups in the surface of the metal hydroxide with ligands from the cell, leading to partial covalent bonds. The preparation of hydrous titanium (IV) oxide is made from the corresponding tetrachloride derivative, which is neutralized to pH 7.0 by

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addition of ammonium hydroxide. The excess ammonium ions are washed with a physiological saline solution from the resulting metal hydroxide, which can then be used as immobilization support. Once the hydrous titanium oxide is added to a cell suspension, cells tend to aggregate and a precipitate is rapidly obtained, which can easily be recovered by centrifugation ***(15)***. This straightforward and easy to follow procedure is hampered however by cell loss and metal leakage ***(15)***. A different cell immobilization procedure involving transition metal chemistry, based on cell adsorption to the support, has also been reported ***(4,5,10)***. Adsorption is a simple and cheap method of cell immobilization, and therefore promising, particularly if a commercial scale process is envisaged. The cell suspension can contact the support in either a stirred tank or can be pumped through a bed of support, to promote cell adsorption. Even the risk of desorption can be turned into an advantage, because the matrix can be easily regenerated, by allowing the cells to desorb, once their catalytic activity decreases ***(15)***.

The support used for cell immobilization can be based on pure titanium (IV) oxide in powder, granular, or honeycomb monolith structure ***(4,5)***. A more conventional support activated with a transition metal salt can also be used ***(10,16)***.

2. Materials

2.1. Immobilization of Yeast Cells on Hydrous Titanium Oxide (1,16,17)

1. Titanium (IV) chloride 150 g/L solution in 150 g/L Hcl (*see* **Note 1**).
2. Ammonium hydroxide 2.0 *M* solution (handle carefully: irritant)
3. *Saccharomyces cerevisiae* cells
4. 0.9% (w/v) Physiological NaCl solution.
5. Fume hood and disposable gloves.

2.2. Immobilization of Arthrobacter simplex *Cells on Titanium-Activated Inorganic Supports*

1. 42 g/L Potato dextrose agar slants.
2. Sterile solution of 10 g/L yeast extract either with or without 0.3 g/L cortisol in 20 m*M* potassium phosphate buffer, pH 7.0.
3. Celite 80–120 mesh.
4. Titanium (IV) chloride 150 g/L solution in 100 g/L HCl (handle carefully: highly corrosive).
5. 20 m*M* Potassium phosphate buffer, pH 7.0.
6. *A. simplex* (ATCC 6946) cells.

2.3. Immobilization of Mycobacterium *spp. Cells Onto Titanium Oxide*

1. 42 g/L Potato dextrose agar slants.
2. Sterile solution of 10 g/L yeast extract in 20 m*M* potassium phosphate buffer, pH 7.0.
3. Sterile solution composed of 10 g/L fructose, 2 g/L ammonium chloride, 0.1 g/L magnesium sulfate, 0.025 g/L calcium chloride, 0.78 g/L Tween-20, and 0.1 g/L sitosterol in 20 m*M* potassium phosphate buffer, pH 7.0.
4. Granular or powdered TiO_2.
5. 20 m*M* Potassium phosphate buffer, pH 7.0.
6. *Mycobacterium* spp. NRRL B-3805 cells.

3. Methods

3.1. Immobilization of Yeast Cells on Hydrous Titanium Oxide

1. Slowly add a 2 *M* ammonium chloride solution to 10 mL of a 150 g/L solution of titanium (IV) chloride until neutrality, pH 7.0 (*see* **Note 2**).
2. Wash the precipitate with 3 × 25 mL of a 0.9% (w/v) NaCl solution. The hydrous titanium oxide is ready to be used as cell support.
3. Add 10 mL of a 2% (w/v) of a yeast cell suspension in 0.9% (w/v) NaCl solution to the support prepared in **step 2**. Stir gently (e.g., 120 rpm in an orbital shaker) at room temperature for 5 min to allow cell immobilization.
4. Allow the mixture to settle down at room temperature, until a clear supernatant is observed.
5. Centrifuge at low speed and discard the supernatant. Determine hydrolytic activity if the immobilized biocatalyst as described in **Subheading 3.2.**

3.2. Determination of the Activity of Yeast Cells Immobilized on Hydrous Titanium Oxide

Activity determination is based on the invertase enzymatic activity of the immobilized cells. This is measured using as substrate a 2.0% (w/v) solution of sucrose in 20 m*M* sodium acetate buffer, pH 4.5.

1. Add 100 mg of immobilized biocatalyst to 5 mL of a 2.0% (w/v) solution of sucrose in 20 m*M* sodium acetate buffer 20 m*M*, pH 4.5, at 45°C.
2. At zero time and at various time intervals along an incubation period of 30 min at 45°C, take 100 µL aliquots and place the samples in an ice bath to stop the reaction.
3. Thaw the samples to room temperature, and add 100 µL of DNS reagent. Mix thoroughly and heat in a boiling water bath for 5 min. Cool to room temperature, add 1 mL of distilled water and measure the absorbance of the solutions at 540 nm. The concentration of the reducing sugars formed may be assessed by using a calibration curve determined according to **Note 3**.

3.3. Immobilization of A. simplex *Cells on Titanium-Activated Inorganic Supports* (10)

1. Cell growth and harvest: *A. simplex* (ATCC 6946) can be grown in 1-L shake flasks containing 250 mL of growth medium, with 150 rpm orbital shaking at 30°C.
2. The growth medium contains 10 g/L of yeast extract in 20 m*M* phosphate buffer, pH 7.0. The inoculum is a 25-mL portion of a 17-h culture on the same medium, grown in the described conditions.
3. Cortisol (inducer of the steroid Δ^1-dehydrogenase) powder is added to the fermentation medium 8 h after inoculation to a final concentration of 0.3 g/L.
4. Cells can be harvested 18 h after induction by centrifugation at 9000 rpm and 10°C and washed twice with a 2% (v/v) methanol solution in 20 m*M* phosphate buffer, pH 7.0.
5. The wet cell paste, containing 220 of cell dry weight/g (determined at 80°C) can be stored at –20°C.

6. Thoroughly wash Celite with distilled water and acetone and dry the support at 80°C overnight.
7. Add 6.5 mL of a 150 g/L solution of titanium chloride in 150 g/L HCl to 1 g of Celite and heat up to 80°C under reflux for about 2.5 h. A hydrous titanium oxide layer is formed that adheres to Celite.
8. Wash thoroughly the support with distilled water and phosphate buffer and filter off with qualitative filter paper.
9. Add 20 mL of a cell suspension, roughly 0.3% (w/v), based on dry cell weight, in 20 m*M* phosphate buffer, pH 7.0, to 1 g of the activated support and stir the mixture for 5 to 7 h, at 30°C and 150 rpm, in an orbital shaker.
10. Periodically monitor the optical density (640 nm) of the supernatant. The cell-loaded support is then filtered off with qualitative filter paper, thoroughly washed with phosphate buffer and used for bioconversion.

3.4. Determination of the Activity of A. simplex *Cells Immobilized on Titanium-Activated Inorganic Supports* (10,18)

The determination of biocatalytic activity is based on the Δ^1-dehydrogenation of cortisol to prednisolone.

1. Add 500 mg of immobilized biocatalyst to 5 mL of a 1.0 g/L solution of cortisol in *n*-decanol (previously saturated with 20 m*M* phosphate buffer, pH 7.0), containing 80 mg/L of menadione (external electron acceptor; *see* **Note 4**).
2. Collect 100 µL samples at time zero and along the time course of the bioconversion up to 24 h and dilute the samples with chloroform containing 120 mg/L cortisone (internal standard) and evaluate the steroid content by HPLC.
3. Steroid analysis is performed by high performance liquid chromatography (HPLC) using a Lichrosorb Si-60 column 250 × 4 mm; 10 µm particle diameter (VWR), with a mixture of (v/v) dichloromethane (94%) methanol (3%) and glacial acetic acid (3%) as mobile phase, at a flow rate of 1.0 mL/min. The products are detected at 254 nm and matched to pure cortisol and prednisolone.

3.5. Immobilization of Mycobacterium *spp. Cells Onto Titanium Oxide* (5)

The determination of biocatalytic activity is based on the side-chain cleavage of sitosterol to 4-androstene-3,17-dione.

1. Titanium oxide must be thoroughly washed with water and acetone and dried overnight at 80°C
2. Cell growth and harvest: cells can be grown in 250-mL Erlenmeyer flasks containing 50 mL of a medium composed of: 10 g/L fructose, 2 g/L ammonium chloride, 0.1 g/L magnesium sulfate, 0.025 g/L calcium chloride, 0.8 g/L Tween-20, and 0.1 g/L sitosterol in 20 m*M* potassium phosphate buffer, pH 7.0. Growth is carried out in an orbital shaker at 30°C and 200 rpm. The inoculum is a 0.5-mL portion of a 17-h culture on yeast extract (10 g/L) in 20 mM potassium phosphate buffer, pH 7.0, grown in the described conditions. Cell adsorption is promoted by adding 2 g of support to the cell culture when an optical density (640 nm) is achieved. Growth is allowed to proceed for a further 4-h period, allowing cell

immobilization to occur. The cell loaded support is then harvested by filtration on qualitative filter paper, thoroughly washed with 20 m*M* phosphate buffer, pH 7.0, and used for bioconversion trials.

3. Dry samples of the cell-loaded support and assay for protein content according to Lowry and co-workers ***(18)***, following cell hydrolysis by heating at 100°C for 20 min in a 1 *M* solution of NaOH ***(19)***.

3.6. Determination of the Activity of Mycobacterium *spp. Cells Immobilized on Titanium Oxide* (5)

1. Add 500 mg of support with immobilized biocatalyst to 5 mL of a 1 g/L suspension of sitosterol in 20 m*M* phosphate buffer, pH 7.0, and incubate in screw-capped bottles placed in an orbital shaker at 200 rpm and 30°C.
2. Collect 100 µL samples at time zero and along the time course of the bioconversion up to 72 h, extract the samples with 400 µL of *n*-heptane containing 200 mg/L of progesterone (internal standard) and evaluate the steroid content by HPLC.
3. Steroid analysis was performed by HPLC using a Lichrosorb Si-60 column 250 × 4 mm; 10 µm particle diameter (VWR), with *n*-heptane containing 5% (v/v) ethanol as the mobile phase at a flow rate of 1.0 mL/min. The products were detected at 215 nm and matched to pure sitosterol and androstenedione.

4. Notes

1. Handle carefully: highly corrosive.
2. Should be performed in a fume hood.
3. To 100 µL aliquots of D-glucose standard solution in a 0 to 1000 mg/L in 20 m*M* sodium acetate buffer, pH 4.5, add 100 µL of DNS reagent, mix thoroughly and heat in a boiling water bath for 5 min. Cool to room temperature, add 1 mL of distilled water and measure the absorbance of the solutions at 570 nm.
4. Incubate in screw capped bottles placed in an orbital shaker at 200 rpm and 30°C.

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Immobilization of Microalgae

Nirupama Mallick

Summary

Several microalgae synthesize metabolites of great commercial interest. Microalgae also act as filters for wastewater N and P, heavy metals, and xenobiotic compounds. However, the cost-effective harvesting of microalgae is one of the major bottlenecks limiting the microalgal biomass applications. In this context, immobilization of algal cells has been proposed for circumventing the harvest problem as well as retaining the high-value algal biomass for further processing. In recent years, innovative approaches have been employed in the field of coimmobilization and microencapsulation, which have proved the superiority of immobilized cells over the free cells. Further, the development in the field of biosensor technology with immobilized microalgae presents an early warning device to monitor pollutants in natural waters. This chapter reviews the various applications of immobilized microalgae and addresses the specific methods concerning the production of coimmobilized beads and the protocol for construction of optical algal biosensors.

Key Words: Coimmobilization; biosensor; bioreactor; heavy metal; microalgae; N and P removal.

1. Introduction

The commercial use of algae has a long history and many species of algae are being used as food, feed, and as sources of valuable chemicals. For example, several species of the macroalgae *Caulerpa*, *Porphyra*, *Iridaea*, *Gigartina*, and *Ulva* are harvested for human food or hydrocolloids ***(1–3)***. Cultivation of *Porphyra*, known as "Nori" in Japan, is one of the largest aquaculture industries in Japan ***(4)***. Moreover, the production of agar, carrageenan, and alginic acid from macroalgae are well-established industries. A few microalgae, such as some species of *Nostoc* and *Aphanizomenon*, are also harvested from the wild for human consumption ***(5)***; however, most commercially used microalgae are cultured. These include *Dunaliella* for β-carotene and *Chlorella* and *Spirulina* for protein production ***(6,7)***.

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Table 1
Basic Requirements of an Efficient Immobilized Algal System and Properties of an Ideal Matrix for Immobilization

Requirements of an efficient immobilized algal system	Properties of an ideal matrix for algal immobilization
Retention of viability	Nontoxicity
Ability to photosynthesize	Phototransparency
High density of cells	Stability in growth medium
Continued productivity	Retention of biomass
Low leakage of cells from matrix	Resistance to disruption by cell growth

From **ref. *15.***

As early as 1949, Spoehr and Milner ***(8)*** suggested that mass culture of microalgae would help to overcome the global protein shortages. The basis for their optimism was that algae had crude protein content in excess of 50%, and biomass productivity of the order of 25 tons/ha/yr. Moreover, N- and P-rich wastewaters are also viewed as a valuable substrate for cultivation of algae ***(9)***. The cultivation of algae in wastewaters offers the combined advantages of treating the wastewaters and simultaneously producing algal biomass, which can further be exploited for protein complements and food additives (for aquaculture, animal and human feed), energies such as biogas and fuels, agriculture (fertilizers and soil conditioners), pharmaceuticals, cosmetics, and other valuable chemicals ***(10)***.

One of the major bottlenecks in microalgal biomass applications is harvesting or separation of algal biomass from the treated water discharge. Numerous efforts have been devoted to developing a suitable technology for harvesting microalgae, ranging from simple sand filtration to energy-intensive centrifugation ***(11–13)***. Autoflocculation (i.e., self-aggregation by stopping aeration followed by decantation), particularly for cyanobacteria, has also been practiced. Harvesting microalgae with ultrasonic waves or ultrasound has also been experimented by Bosma et al. ***(14)***, where the separation process is based on gentle acoustically induced aggregation followed by enhanced sedimentation. In this context, immobilization of algal cells has been proposed for circumventing the harvest problem as well as retaining the high-value algal biomass for further processing. **Table 1** summarizes the basic requirements of an efficient immobilized algal system and the properties of an ideal matrix for immobilization.

1.1. Immobilization Techniques

Although several methods are available for immobilization of cells and enzymes, entrapment is by far the most frequently used method for algal immobilization. Several natural (e.g., collagen, agar, agarose, cellulose, alginate, carrageenan) and synthetic (e.g., acrylamide, polyurethane, polyvinyl) polymers are used for this purpose. However, for algal immobilization the most frequently used natural gels are alginate and carrageenan. The gel is generally formed into useful biocatalyst beads by first adding the algal cells as a suspension to an aqueous solution of the

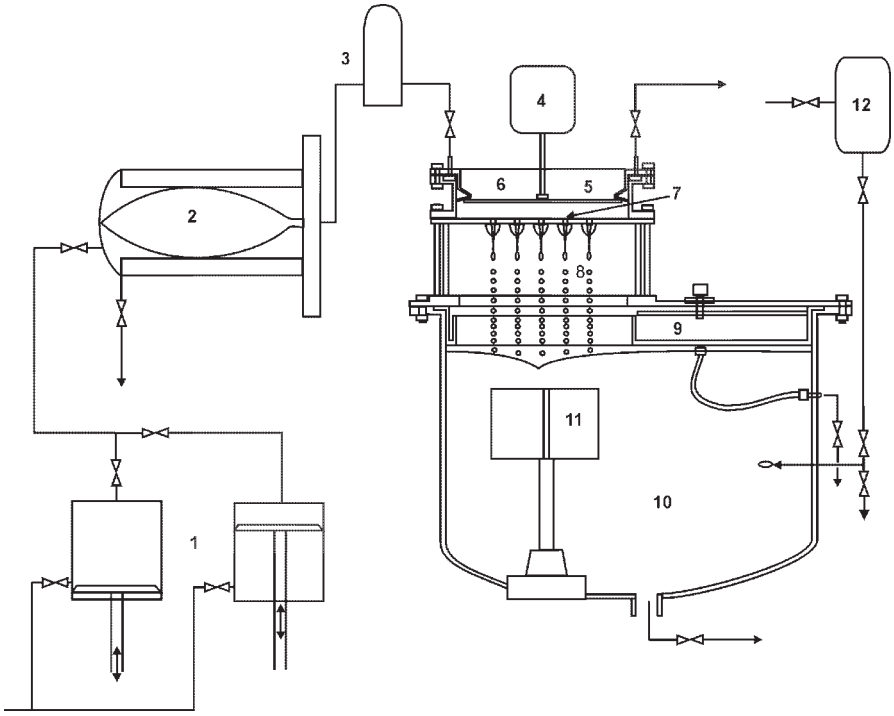

Fig. 1. Schematic representation of the multinozzle system: *(1)* double piston pump; *(2)* sterile barrier; *(3)* damper; *(4)* vibrator; *(5)* membrane of pulsation chamber; *(6)* concentric split; *(7)* pulsation chamber; *(8)* nozzle plate; *(9)* bypass system; *(10)* reaction vessel; *(11)* stirrer, and *(12)* input hardening solution. (From **ref.** ***16*** with permission.)

gelling material. This material is then formed into droplets by forcing it dropwise through a nozzle or orifice to an interacting salt solution. The droplets are subsequently stabilized into biocatalyst beads with the entrapped organisms via polymerization or other types of cross-linking. For example, alginate droplets can be stabilized with divalent ions such as Ca^{2+}, and carrageenan droplets are typically cross-linked with K^{+}. A new multinozzle system with thirteen nozzles for the encapsulation of microorganisms, enzymes, or cells was developed by Brandenberger and Widmer ***(16)*** *(see* **Fig. 1**). Based on the laminar jet break-up, monodispersed beads of calcium alginate are produced under sterile and reproducible conditions in the range of 0.2 to 1.0 mm. An *in situ* cleaning of the nozzles is implemented in order to guarantee several batch process cycles and a productivity of up to 5 L/h. Beads were analyzed and showed that the relative difference of the mean diameter of different nozzles was less than 0.3%.

Most recently however, coimmobilization of microalgae with the bacterium *Azospirillum brasilense* was reported, which was found to improve growth, pigment and lipid contents, and cell and population size of the entrapped microalgae ***(17)***. This novel coimmobilized biocatalyst was also found superior for removal of

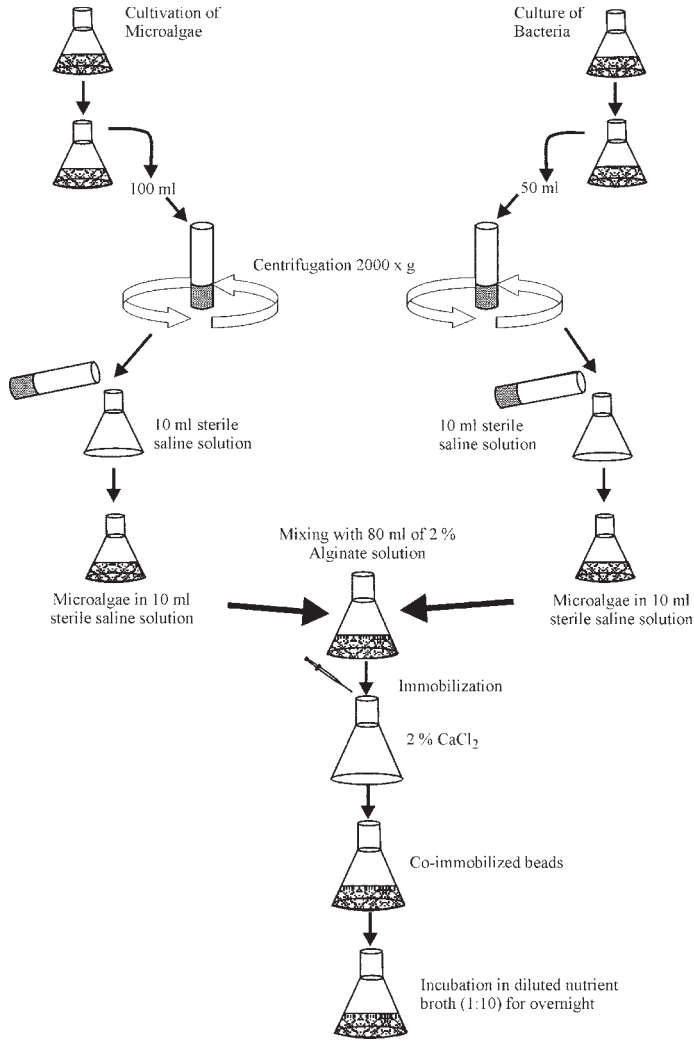

Fig. 2. Flow chart showing methods used to coimmobilize microalgae and MGPB. (From **ref.** ***17*** with permission.)

N and P rather than by the immobilized-algae alone. The device for coimmobilization is discussed in **Subheadings 2.–4.**, and the flow chart is presented in **Fig. 2**.

Since 1990, 50% of all reports dealing with immobilized microalgae have involved their use in wastewater N and P removal, and others on the use of immobilized algae for metal accumulation and removal. Most recently, however, novel conductometic biosensors are developed using immobilized microalgae ***(18–21)***. These fiber optic biosensors are used for monitoring toxic compounds such as

heavy metals, herbicides, etc. in the environment. Developmental procedure for this biosensor is described in **Subheadings 2.–4.**, and the device is shown in **Fig. 3**.

1.2. Application of Immobilized Algae

1.2.1. Production of High-Value Products

Reports showed that immobilization has a significant impact on algal cell productivity. Hydrogen evolution from the filamentous alga *Anabaena* was reported to increase threefold following immobilization ***(22,23)***. Brouers and Hall ***(23)*** and Bailliez et al. ***(24)*** demonstrated an enhanced production of ammonia and hydrocarbon, respectively, by *Mastigocladus laminosus* and *Botryococcus* spp. following immobilization. Santos-Rosa et al. ***(25)*** also found that *Chlamydomonas reinhardtii* cells immobilized in Ba-alginate provide a stable and effective system for photoproduction of ammonia. Glycolate production in alginate-entrapped cells was shown to be double that in the free-living cells ***(26)***. Leon and Galvan ***(27)*** studied the production of glycerol in *C. reinhardtii* cells immobilized in Ca-alginate. The immobilized cells registered a production rate of 7 g/L in comparison to 4 g/L by their free-living counterparts.

Efficiency of immobilized cells and thylakoid vesicles of the microalga *C. reinhardtii* CW-15 were compared with its free-living counterparts for production of hydrogen peroxide ***(28)***. H_2O_2 is an efficient and clean fuel used for rocket propulsion, motors, and for heating. This compound is produced by the photosystems in a catalytic cycle in which a redox mediator (Me viologen) is reduced by the electrons obtained from water. Under optimum conditions, the photoproduction, a rate of 33 µmol H_2O_2/mg *Chl.*/h was maintained for several hours by Ca-alginate entrapped cells with an energy conversion efficiency of 0.25%. As compared with this the immobilized cyanobacteria (*Anabaena variabilis* and *Anacystis nidulans*) with poor catalase activity depicted a productivity of 150 and 60 µmol H_2O_2/mg *Chl.*/h, respectively ***(29,30)***.

Rossignol et al. ***(31)*** entrapped the benthic diatom, *Haslea ostrearia* in agar as well as alginate beads, and studied it for marennine production. With identical conditions, the specific productivity of marennine was higher using a photobioreactor with immobilized cells rather than free cells. Based on this study a new photobioreactor has been designed by Lebeau et al. ***(32)*** for marennine production (*see* **Fig. 4**). Most recently, whole-cell immobilization of the microalgae *Botryococcus braunii* and *B. protuberans* in alginate beads under airlift batch culture resulted in significant increase in hydrocarbon production at the resting phase of growth ***(33)***.

1.2.2. Removal of N and P

A large number of reports are published on the use of immobilized microalgae for wastewater N and P removal (i.e., for the tertiary treatment of wastewater). In general, immobilized cells are found more efficient in removing N and P as compared to their free-living counterparts, and removal of phosphate is a much slower process than that observed for nitrogen ***(15)***. Further, a gradual decline in efficiency from the first to subsequent cycles was also observed. However, in 1986, Jeanfils and Thomas ***(34)*** with alginate-immobilized *Scenedesmus obliquus* demonstrated that nitrite uptake efficiency was not affected by immobilization, except

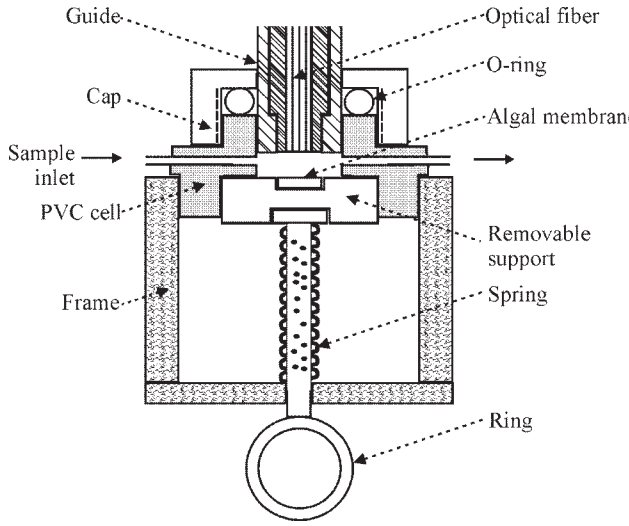

Fig. 3. Device for an algal optical biosensor. (From **ref.** ***18*** with permission.)

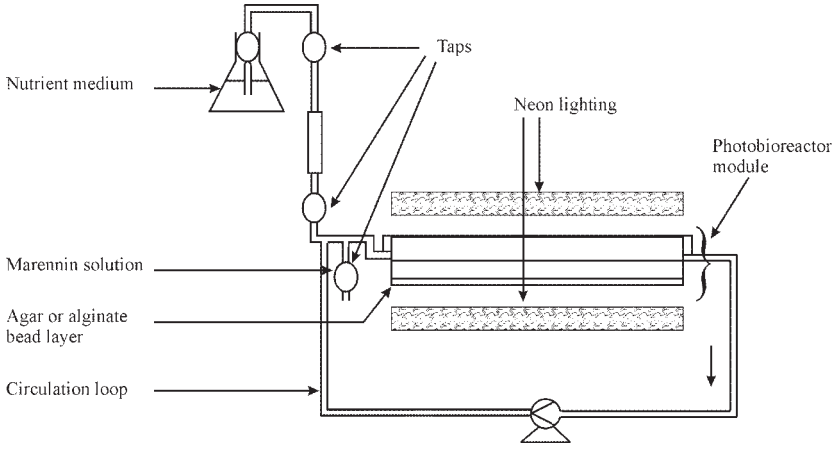

Fig. 4. Photobioreactor used for marennin production. (From **ref.** ***32*** with permission.)

that a longer lag phase was observed in immobilized cells than the free ones. Megharaj et al. ***(35)*** provided evidence for the insignificant impact of culture age on nitrogen removal. In contrast to this, batch culture studies of phosphate uptake by alginate-entrapped *Chlorella emersonii* have shown that the exponentially growing cells remove phosphate from the medium five times more rapidly than the cells in the late stationary phase. When cells of different ages are immobilized in Ca-alginate and packed in a small-scale packed-bed reactor the effects of culture age are sufficient to produced significant differences in reactor performance

(36). Nitrogen starvation however, greatly increased the N uptake rate of immobilized *S. obliquus* ***(37)***.

Rai and Mallick ***(38)*** demonstrated a higher uptake rate for both N and P by immobilized *Chlorella* and *Anabaena* than their free-living counterparts. In subsequent studies Mallick and Rai ***(39,40)*** also observed that immobilized algae with a cell density of 0.1 g dry wt/L was the most efficient for nutrient and metal removal in a pH range of 6.0 to 8.0, and chitosan could be a promising algal support for wastewater detoxification. Vilchez and Vega ***(41)*** found that alginate-entrapped *C. reinhardtii* cells provide a stable and functional system for removing nitrogenous contaminants from wastewater. A group of parameters such as matrix concentration, cell loading, temperature, and pH were also considered in order to determine the best working conditions for the immobilized cells. In case of *C. reinhardtii* cells an alginate concentration of 3% is adequate for minimizing substrate diffusion problems, thereby allowing the beads to attain a physical consistency.

Garbisu et al. ***(42)*** studied phosphate removal efficiency of foam-immobilized *Phormidium laminosum* both in batch and continuous flow fluidized-bed bioreactors. Fluidized-beds were designed as funnel-shaped, column-type beds and beds in Erlenmeyer flasks. The bioreactors were continuously illuminated with cool white fluorescent lamps at an irradiance of 100 μmol photon m^2/s and were operated at 45°C by submerging the lower part of the bioreactor in a thermostatically controlled water bath. Preboiled, washed, and dried 5-mm foam cubes were placed in algal suspension and immobilization by adsorption was continued over 2 mo. Once the foam cubes were fully colonized, they were introduced into the bioreactors. Although cyanobacteria immobilized on the polymer foams did remove N and P from the system efficiently, the fluidized-bed reactors resulted in a heterogeneous system, which is unsuitable for most laboratory standardization. Several workers however, studied removal of N and P in packed-bed reactors. Poor light penetration, nonmixing of cells, and gaseous fluxes are limitations in operation of this type of reactors.

Kaya and Picard ***(43)*** developed a novel immobilized algal system for wastewater biotreatment. In this new process the green microalga *Scenedesmus bicellularis,* isolated from a secondary decantation tank, was grown in a synthetic medium for 12 d, and the cells collected after centrifugation were immobilized on alginate screens. The screens were inserted into a photochamber saturated at 100% relative humidity and a photoperiod of 16 h with an illumination of 150 μE m^2/s (*see* **Fig. 5**). After 48 h of nutrient starvation, the immobilized cells were used for the removal of ammonium and orthophosphate from a synthetic secondary wastewater effluent in a flexiglass reactor. In a subsequent study Kaya et al. ***(44)*** also demonstrated that intermittent CO_2 enrichment accelerates tertiary wastewater treatment. However, one major problem with the use of gel materials is maintaining the integrity of alginate gels beyond a few weeks. Although agarose gel-based reactors showed comparatively more stability than others, these polysaccharide-based matrices are very much prone to microbial attack when placed in natural environment. To overcome these difficulties, Robinson ***(45)*** proposed a new type hollow-fiber reactor. Hollow-fiber cartridges are commercially available in a variety of sizes. Robinson ***(45)*** prepared the hollow-fiber reactor with 50 cylindrical

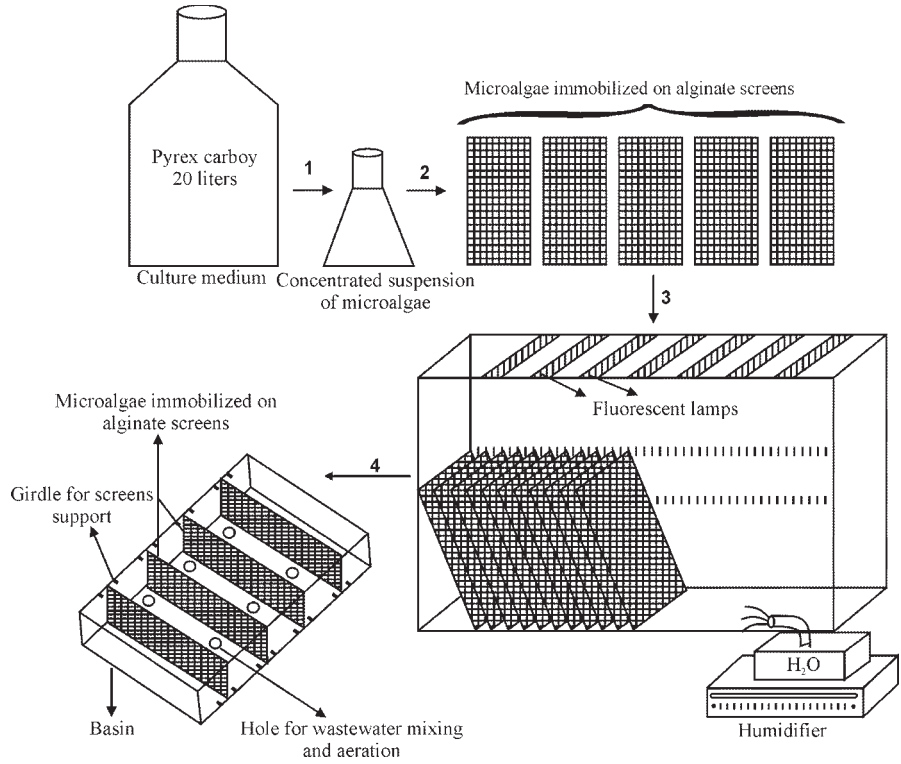

Fig. 5. Schematic description of wastewater treatment by microalgae immobilized on screens and starved in air, saturated at 100% relative humidity: (1) centrifugation; (2) immobilization; (3) starvation in air saturated at 100% relative humidity; and (4) incubation procedure in wastewater. (From **ref.** ***43*** with permission.)

tubes of polysulfone, bundled and sealed in a transparent cartridge of 20 cm in length. Each fiber was of 1.1-mm internal diameter (id) and the fiber wall was perforated by numerous pores. Such a cartridge could be operated with algal cells contained within the lumen of each fiber and the nutrients pumped in through the shell space or vice versa. Preliminary results showed that phosphate uptake rate declined within a matter of hours, not resulting from any lack of activity but as a result of biomass settling within the reactor. However, when cells were suspended in a solution of 1% Na-alginate, settlement rates were significantly slow.

Sawayama et al. ***(46)*** designed a tubular photobioreactor with the thermophilic cyanobacterium, *Phormidium laminosum*, immobilized on cellulose hollow fibers. The removal of nitrate and phosphate from wastewater was studied at 43°C by continuously supplying dilute growth medium for 7 d and then secondarily treated sewage for 12 d. The removal of nitrogenous and phosphate ions from secondary-treated sewage were 0.24 and 0.11 mmol/d/L, respectively, under the same conditions. A wastewater purification system using thermophilic cyanobacteria has

special advantage since contamination can be avoided because of their ability to tolerate high temperature.

Most recently, de-Bashan et al. ***(17,47)*** developed a coimmobilized system (a combination of microalgae, *Chlorella vulgaris* or *Chlorella sorokiniana* and a microalga growth-promoting bacterium (MGPB, *Azospirillum brasilense* strain Cd) to remove nutrients (P and N) from municipal wastewater. *A. brasilense* significantly enhanced the growth, pigment and lipid content, and cell and population size of both the *Chlorella* species when coimmobilized in the small alginate beads ***(47)***. Coimmobilization of the two microorganisms was found superior for removal of N and P than by the immobilized-microalgae alone, reaching removal of up to 100% ammonium, 15% nitrate, and 36% phosphorus within 6 d (varied with the source of the wastewater), compared to 75% ammonium, 6% nitrate, and 19% phosphorus by the microalgae alone ***(17)***, thus showing the potential of coimmobilization of microorganisms in small beads to serve as a treatment system for wastewater.

1.2.3. Metal Removal

One of the main interests for microalgae in biotechnology is focused on their use for heavy metal and radionuclide removal from effluents and wastewaters. In parallel to detoxification, it is also possible to recover valuable elements such as gold, silver and uranium after appropriate treatment of the loaded algal biomass ***(48)***.

Several reports are available on the accumulation of metals by immobilized microalgae (**Table 2**). The most interesting of them is the preparation of "AlgaSORB" by Darnall ***(50)*** of New Mexico State University. In this process the algae are packed in a column-shaped matrix of solid silica gel. The process of packing the algae in the solid matrix kills the microorganisms. Nevertheless, their cell walls still provide a plentiful source of binding sites, which can hook heavy metal ions from the solution. Subsequently, the removal of mercury from aqueous solution by a packed-bed reactor (PBR) containing *Chlorella emersonii* entrapped in alginate and agarose gels was studied ***(54)***. Reactors were constructed from chromatography columns packed with 200 gel particles of 4- to 6-mm in diameter. The effects of variation in cell stocking density, influent mercury concentration, and flow rate on mercury removal were investigated. It was found that levels of mercury volatilization could be reduced by using agarose rather than alginate as the immobilizing matrix. Lau et al. ***(55)*** designed a laboratory scale algal column reactor with the green microalga *Chlorella vulgaris* with 75 mL alginate-algal beads and was used to treat 30 mg/L Cu and Ni with a hydraulic retention time (HRT) of 30 min. At the end of loading 4 L metal solution, over 97% of Cu and 91% of Ni were removed from the wastewater. Up-flow was preferred to down-flow in maintaining a constant flow rate.

Torresday-Gardea et al. ***(56)*** immobilized the biomass of *Synechococcus* sp. *PCC7942* in silica-polymer and the metal binding ability was studied under continuous flow conditions. Results showed maximum adsorption for Pb followed by Cd, Cu=Ni. 0.2 *M* HCl was found effective for recovery of the adsorbed metals. Experiments were conducted to determine if many cycles of metal binding/stripping by the immobilized biomass were possible. Cd, Cu, and Ni were found to be sorbed and desorbed three times, whereas for Pb as many as six times was

Table 2
Summary of Literature on Metal Accumulation by Immobilized Algae

Algal genera	Type of treatment	Immobilizing matrix	Name of the metal	Ref.
Chlorella emersonii	Batch	Alginate	Hg	***49***
Algae (nonspecific)	PBR	Silica	Metal (nonspecific)	***50***
Chlorella homosphaera	Batch	Alginate	Cd, Zn, and Au	***51***
Chlorella salina	Batch	Alginate	Co, Zn, and Mn	***52***
Chlorella salina	Batch	Alginate	Cs	***53***
Chlorella vulgaris and *Anabaena doliolum*	Batch	Alginate	Cu, Ni, and Fe	***38,39***
Chlorella vulgaris and *Anabaena doliolum*	Batch	Alginate, carrageenan, agar, chitosan	Ni & Cr	***40***
Chlorella emersonii	PBR	Alginate, agarose	Hg	***54***
Chlorella vulgaris	PBR	Alginate	Cu & Ni	***55***
Synechococcus sp. PCC 7942	PBR	Silica	Cu, Ni, Pb and Cd	***56***
Chlorella vulgaris	FBR	Alginate	Cu	***57***
Scenedesmus acutus, *Chlorella vulgaris*	FBR, PBR	Polyurethane, κ-carrageenan	Cd, Cr & Zn	***58***
Spirogyra sp.	PBR	Silica	Cu	***59***
Nannochloropsis gaditana	PBR	Alginate	Cu & Zn	***60***
Chlorella sorokiniana	PBR	*Luffa* sponge	Cd	***61***
Chlorella sorokiniana	PBR	*Luffa* sponge	Ni	***62***

PBR, packed-bed reactor; FBR.fluidized-bed reactor.

reported. Travieso et al. ***(58)*** compared the metal removal efficiency of *Scenedesmus acutus* and *Chlorella vulgaris* immobilized in polyurethane foam and κ-carrageenan gel in fluidized- and packed-bed reactors. Immobilized cells are found tolerant to Cd, Cr, and Zn than free cells, thus implying their great possibility for wastewater treatment processes.

In 2000, Singh and Prasad ***(59)*** prepared one "AlgaSORB" column taking silica, a polymer matrix (poly-*N*-xylene-*N*, *N*'-dicyclohexylethylenediamine dibromide) and a green microalga (i.e., *Spirogyra)* which showed unique ion selectivity for Cu from a host of diverse metal ions of the waste samples. Effects of flow rate, pH, equilibrium time, and other variables are also standardized. It has favorable kinetics without showing any clumping/clogging/leaching complications of the stationary support. This system has attractive features for application in single-column ion-chromatography with high durability, simplicity, and economy.

A new sorption system of *Chlorella sorokiniana* immobilized on the biomatrix of a vegetable sponge (*Luffa cylindrica)* was also tried for removal of Cd and Ni from the contaminated aqueous medium in continuous liquid flow column ***(61,62)***. As usual the immobilized *Chlorella sorokiniana* showed significantly higher efficiency than its free-living counterparts. Metal desorprtion with 0.1 *M* HCl was 100% and the desorbed immobilized system was found reusable with similar efficiency for the second cycle. This microalga-luffasponge-immobilized-disks (MLIDs) retained about 93% of the initial binding capacity for Ni up to five cycles of reuse. Thus, because of their high efficiency, low cost, simplicity of preparation, and repeated use for several cycles the MLIDs could provide an attractive high-affinity biosorption system for heavy metal removal.

Volesky and Prasetyo ***(63)***, however, used a new biosorbent material derived from the marine brown alga, *Ascophyllum nodosum*, in a packed-bed flow-through column. Removal of Cd was found to reach 99.98% from an effluent containing 10 mg Cd/L. Rai and co-workers (Banaras Hindu University, Varanasi, India) have studied the metal removal efficiency of the bloom-forming cyanobacterium *Microcystis*. *Microcystis* is an abundantly occurring nuisance cyanobacterium in many eutrophic ponds and reservoirs of India and other tropical countries. This cyanobactrium occurs in a natural immobilized state due to presence of a capsule or slime layer around the cell. Biosorption was found to be influenced by pH and temperature ***(64)***. Interestingly however, heat-killed, formaldehyde-treated, and metabolically inactive (DCMU-treated) *Microcystis* had the same biosorption potential as the metabolically active cells ***(65)***. This suggests that even the dead biomass could be equally useful for metal removal. This group is currently working on the feasibility of using the dried biomass for large-scale application of *Microcystis* as an "AlgaSORB" for metal accumulation removal.

1.2.4. Biosensor Development

Recent interest in immobilized microalgae in biotechnology is focused on the development of biosensors for detection of hazardous chemicals such as heavy metals and pesticides in the environment. In 2000, Naessens and co-workers ***(18)*** developed a biosensor with the microalga *Chlorella vulgaris* immobilized on a removable membrane and placed in front of the tip of an optical fiber bundle inside a homemade microcell (the detailed procedure for construction of a biosensor

is described in **Subheadings 2.–4.**). The detection of herbicides was based on the kinetic measurement of chlorophyll fluorescence. A continuous culture was set up to produce algal cells in reproducible conditions for measurement optimization. Effects of flow rate, algal density, temperature, and pH on biosensor response to various herbicides were studied. Detection of PSII herbicides was achieved at sub-ppb concentration level ***(20)***. However, for detection of heavy metals a biosensor is constructed based on the activity of alkaline phosphatase (AP), present on the external membrane of *Chlorella vulgaris* ***(19)***. It appeared that these conductometric biosensors were more sensitive than bioassays to detect low levels of cadmium ions (the detection limit for Cd was 1 ppb). The main advantage of these AP-based biosensors was their high stability, because contrary to enzymatic biosensors they use whole algal cells with APs on their cell walls ***(21)***. The enzyme remains in its natural environment which favors long-term stability and reflects the mechanism of toxic inhibition, being therefore of ecological interest.

1.3. Concluding Remarks

Biotechnology of immobilized microalgae, though not a subject of current augmentation, has gained importance in recent years as a result of the development of some new production systems and environmental technologies. It is quite clear that a combination of solar energy utilization and algal immobilization can be advantageously applied in the development of photo-activated systems for the treatment of wastewaters and production of valuable algal products. However, some preliminary reports have indicated that immobilization affects the cell's behavior and reduces productivity ***(66,67)***. But recent development in the field of "microencapsulation" has clearly proven the superiority of cell immobilization over the free cells. Under the batch culture of aerobic conditions, the thickness of the capsule membrane and CO_2 supply did not affect the growth of microalgae. The cell concentrations of microencapsulated *Dunaliella bardawil* and *Haematococcus pluvialis* were found to be five times greater than that of the free cells ***(68)***. Based on these results, microencapsulation for the culture of microalgae was suggested as an effective method for the high-division cultivation, and thus higher production. Further, current approach on coimmobilization of microalgae with the bacterium *Azospirillum brasilense* strain Cd was found to enhance growth and increase synthesis of pigments and lipids in three test *Chlorella* species ***(47)***, thus clearly indicating a significant change in the metabolism of the microalgae in presence of this growth-promoting bacterium. However, this new coimmobilzation system is capable of reducing the nutrient load at a much higher rate than the immoblized algae alone from the regular municipal wastewater ***(17)***. Although the removal rate is still under 1 mg/L, this coimmobilization system provides a new approach to the biological removal of nitrogen and phosphorus from wastewater.

Recent developments in the field of biosensor technology with immobilized microalgae, either those detecting the presence of toxic metals by inhibition of alkaline phosphatase activity or those measuring chlorophyll fluorescence for detection of PSII-inhibiting herbicides, are found to be highly sensitive, as detections are observed at sub-ppb level. These chlorophyll fluorescence-/AP-based biosensors deliver fast and reproducible signals that detect some herbicides below regulation concentrations limits. The immobilization method provides inexpen-

sive, ready-made membranes that are easy to handle, and thus this optical algal biosensor could be used as an early-warning device to monitor the target pollutants in natural waters. For environmental management, these small and quick-answering biosensors can be considered as competitive *in situ* tools as soon as the effects of pollutants on the system can be reversed, which means the membranes can be reusable after reversion. Studies led on AP activity showed the efficiency of ethylene diamine tetraacetic acid (EDTA) for reversing the action of some heavy metal ions ***(69,70)***. However, examining to what extent this is also possible in the complex environmental samples is an important task for future investigation.

2. Materials

2.1. For Coimmobilized Bead Preparation

1. *Azospirillum brasilense* strain Cd DMS 1843.
2. Axenic *Chlorella* cultures.
3. Sterile mineral medium (C30; *see* **Note 1**).
4. Nutrient broth (*see* **Note 2**).
5. Sterile saline solution: 0.85% NaCl solution (w/v).
6. Alginate solution: 2% Na-alginate (w/v).
7. $CaCl_2$ solution: 2% (w/v).

2.2. For Preparation of Algal Optical Biosensor

1. *Chlorella vulgaris* strain CCAP 211/12.
2. Glass microfiber filter GF/C, 45.7-mm filter diameter, 1.2 mm pore diameter (Whatman, England, information@whatman.com).
3. Flow cell (homemade; *see* **Fig. 3**).
4. Fluorometer equipped with a microcomputer.
5. 0.1 *M* Tris-HCl buffer, pH 8.5.
6. 1 m*M* $MgCl_2$.
7. 0.1 m*M* Methylumbelliferoyl phosphate (MUP).

3. Methods

3.1. Procedure for Production of Coimmobilized Beads (17)

1. Cultivation of axenic microalgal cultures in sterile mineral medium.
2. Culture *Azospirillum brasilense* strain Cd in liquid nutrient broth.
3. Take 100 mL of the axenic microalgal culture containing approx 6×10^6 cells/mL, wash twice in sterile saline solution (0.85% NaCl), and resuspend in 10 mL of same sterile saline solution.
4. Take 50 mL of *A. brasilense* culture, wash twice in sterile saline solution, and resuspend in 10 mL of saline solution (the optical density should be adjusted to 1 at 540 nm to have approx 10^9 CFU/mL).
5. To coimmobilize the two microorganisms in the same bead, mix both the cultures (20 mL volume).
6. Now mix the 20 mL of cultures with 80 mL of a sterile 2% alginate solution and stir slowly for 15 min.
7. Drop the solution from a syringe into a 2% $CaCl_2$ solution and leave the beads for 1 h in the solution for stabilization (*see* **Note 3**).

3.2. Preparation of Optical Biosensor Using Immobilized Microalgae (18,19)

3.2.1. Preparation of Microalgal Membrane

1. Cultivate the microalgae (for example *Chlorella vulgaris* strain CCAP 211/12) in sterile culture medium.
2. To induce high AP activity, centrifuge the algal cultures and resuspend the pellets in phosphate-free medium for 25 d, which corresponds to maximal enzyme activity. But for the biosensors based on chlorophyll fluorescence measurement cultivate the algae under continuous mode (*see* **Note 4**).
3. Filter 2 mL of the resulting aliquot on a glass microfiber filter (GF/C, Whatman).
4. Dry the filter for 2 h at 105°C.
5. Keep the glass microfiber filter in a desiccator for 30 min before fitting to the optical transducer.

3.2.2. Construction of Algal Optical Biosensor

1. Place the algal membrane in a 1-mL flow cell (*see* **Fig. 3**).
2. Place a bifurcated bundle of randomized optical fibers opposite to the membrane. The orientation should be in such a way that the incident light after hitting the upper part of the membrane should be transmitted to the Fluorometer.

3.2.3. Operation of Algal Optical Biosensor

1. Test the biosensor by adding the MUP solution into the microplate wells containing *Chlorella vulgaris* (*see* **Notes 5** and **6**). Testing can also be done by measuring chlorophyll fluorescence directly.
2. Feed the sensor with toxic-free solution, called reference, at first.
3. Then introduce herbicide/heavy metal solution and measure the biosensor response.
4. Analyze the response of the sensor to pollutants by comparing the peak heights.
5. After running the toxic compounds, reversibility of the biosensor should be tested each time (s*ee* **Note 7**).

4. Notes

1. Sterile mineral medium composition:
   KNO_3 : 25g, $MgSO_4 \cdot 7H_2O$: 10 g, KH_2PO_4: 4 g, K_2HPO_4: 1 g, $FeSO_4 \cdot 7H_2O$: 1 g, H_3BO_3: 2.86 µg, $MnCl_2 \cdot 4H_2O$: 1.81 µg, $ZnSO_4 \cdot 7H_2O$: 0.11 µg, $CuSO_4 \cdot 5H_2O$: 0.09 µg, Na_2MoO_4: 0.021 µg/L. Axenic *Chlorella* cultures were incubated for 5–6 d under continuous agitation (150 rpm), light intensity of 31.8 W/m^2 , and at 27–30°C.
2. *Azospirillum brasilense* strain Cd DMS 1843 was grown in liquid nutrient broth (Sigma) or M9 nitrogen-free medium (composition g/L: Na_2HPO_4: 6 g, KH_2PO_4: 3 g, NaCl: 1.5 g, $MgSO_4 \cdot 7H_2O$: 1 *M*; $CaCl_2$: 0.01 *M*, malic acid: 20%) at 30 ± 2°C and agitation (120 rpm) for 17 h.
3. As immobilization normally reduces the number of bacteria in the beads, incubate the beads overnight in diluted nutrient broth (1:10). After washing thrice in sterile solution the beads are ready for application.
4. To date, operations of algal biosensors are based on two major principles. In the first type, the operating principle is based on the inhibition of the activity of AP. AP activity is determined with methylumbelliferoyl phosphate (MUP) as sub-

strate, which is converted into methylumbelliferone (MUF), a fluorescent compound by the action of alkaline phosphatase.

$$\text{MUP} \xrightarrow{\text{AP}} \text{MUF (methylumbelliferone)} + \text{P}$$

AP activity can easily be measured by measuring the MUF production at fluorescence emission of 460 nm under excitation light of 350 nm.

The second type biosensor however, is based on algal chlorophyll fluorescence measurement at excitation of 482 nm and emission of 683 nm.

5. The substrate MUP was placed in the Tris buffer solution (0.1 *M*, pH 8.5) containing 1 m*M* $MgCl_2$ as enzyme activator, and AP activity was measured in terms of fluorescence intensity.
6. AP activity can also be measured with another substrate, *p*-nitrophenyl phosphate (pNPP). But preference should be given to MUP for its higher sensitivity.
7. After running the toxic solutions in the biosensor, it should be clean thoroughly and reversibility of the biosensor should be tested by running the reference solution each time.

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Bioluminescence in Immobilized Cells for Biomass Detection and Biosensor Applications

Marián Navrátil, Juraj Švitel, and Peter Gemeiner

Summary

Bioluminescence measurements have become extremely popular because of good sensitivity and the ability to quantitate a wide variety of analytes. Only recently have these measurements gained particular interest in relation to the study of gene expression and regulation. The firefly luciferase system is the most frequently used bioluminescent reaction, in which adenosine triphosphate (ATP) is used to generate light. ATP is often used as a measure of cell biomass, because the intracellular level of ATP is rather constant and is rapidly degraded upon cell death. The bioluminescence principle has been used successfully in a variety of biosensor applications, mostly for the environmental monitoring. In this chapter we describe detection of viable biomass in free and immobilized cells and outline a few applications, where this approach can be used for biosensor construction. We demonstrate the advantages of bioluminescence by two protocols for detection of ATP and viable biomass in free and immobilized cell samples, which have been proved very rapid and accurate. This indicates that bioluminescence measurements are a good indicator of cell viability and can be widely applied in a number of areas such as biotechnology and food industry.

Key Words: ATP; bioluminescence; biosensors; cell viability; entrapment; immobilization; luciferase.

1. Introduction

1.1. Principles of Bioluminescence

The phenomenon of bioluminescence was first found more than a century ago in fireflies and the enzyme which catalyzes the oxidative light-emitting reaction was named luciferase. The biochemical reaction in its simplest form can be written as:

$$\text{Luciferin} + O_2 \xrightarrow{\text{luciferase}} \text{Oxyluciferin} + CO_2 + \text{AMP} + \text{light} \qquad (1)$$

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Later many other bioluminescent organisms were found, including insects, fish, and bacteria. The luciferase reaction shown in **Eq. (1)** uses adenosine triphosphate (ATP) to generate light, however marine luminescent bacteria use reduced flavine instead of ATP. Bioluminescent bacteria can be found in nature in different habitats ranging from marine to terrestrial environments. In addition to naturally occurring microorganisms also genetically engineered microorganisms are used to develop bioluminescence-based biosensors. Gene coding enzymes needed for bioluminescence were mapped, isolated, and cloned. Genetic technology now enables the transfer of bioluminescence to a wide variety of microorganisms. Genetically engineered strains harboring bioluminescent reporter plasmids are now of growing importance for development of microbial luminescent biosensors ***(1)***.

The very early report of analytical application of bioluminescence appeared almost a hundred years ago, when Beijerinck described the detection of photosynthetically formed oxygen in leaf extract, using luminuous bacteria. The phenomenon of bioluminescence was exploited to develop a wide variety of bioluminescent and chemiluminescent biosensor techniques. Bioluminescence found its practical applications mainly in development of instrumental methods in:

1. Measurement of microbial and other cells based on ATP content measurement.
2. Determination of active immobilized biomass including, and
3. Bioluminescence-based biosensors, mainly for environmental applications.

1.2. Bioluminescence-Based Biomass Measurement

Bioluminescence principle has been extended from detection of luminescent cells to the detection of naturally nonluminescent organisms. Intracellular ATP, and possibly also other nucleotides, can be used to generate light after light-generating enzymes are being added to the system. The first works dealing with determination of biomass using bioluminescence principle are more than 30 yr old. Intracellular ATP content was studied in 19 species of *Escherichia coli* in detail and was found in the range $0.28–8.9 \times 10^{-10}$ μg of ATP per organism ***(2)*.** In principle, cell detection is conducted in vitro; it is based on efficient extraction of intracellular ATP with minimal ATP hydrolysis. Subsequently, light generating system is added (luciferin + luciferase) and emitted light is measured. Numerous agents to extract ATP have been tested, including organic solvents, inorganic acids, and surfactants. Therefore, the analysis of ATP can be utilized as a method to detect microbial cells at very low concentration. The method was adapted to the detection of various types of cells: bacterial content in fluids ***(2)***, protozoa ***(3)***, mycobacteria ***(4)***, yeasts ***(5)***, tumor cells ***(6)***, and other tissues. Luciferin and luciferase required for the assay are commercially available, as well as commercial luminometers or other light-detecting instrumentation. A survey on commercially available luminometers and imaging devices for low-light level measurements was recently compiled ***(7)***.

Other methods use direct monitoring of cell number and their metabolic activity with incorporated chromosomal *luxAB* and *gfp* genes, encoding bacterial luciferase and green fluorescent protein, respectively. Because the metabolism requires energy, bioluminescence output is directly related to the metabolic activity of the cells ***(8)***.

1.3. Bioluminescence-Based Determination of Immobilized Biomass

The luminometric method for the determination of active immobilized biomass concentration is also based on measuring ATP content in cells. ATP serves as a carrier of chemical energy in cells, where available energy is stored in chemical bonds between terminal phosphate groups. After cell death, the ATP concentration rapidly decreases. Because ATP is a good indicator of cell viability, its concentration is dependent on active biomass amount ***(9)***. The concentration of ATP can conveniently be determined using bioluminescence method, as described above.

Extraction of the intracellular ATP is critical for a successful measurement. There are several extraction methods, and the choice of extractant depends on a particular microorganism. The extraction procedure should achieve quantitative release of nucleotides and simultaneously inactivate any nucleotide-converting enzymes which may be present in the immobilized cells. Wijffels et al. proved, that 90% of the living cells immobilized by entrapped in gel beads are located in a 140-μm thick outer layer of a 1-mm bead ***(10)*** and the cells retained more than 90% of their activity after the immobilization ***(11)***. Thus, the extraction of ATP from immobilized cells has been shown as quite effective.

Although attempts to measure free biomass by bioluminometry are widely described, only a few reports on bioluminescence-based estimation of immobilized biomass exist. The content of *Beneckea natriegens* cells immobilized on silica particles was tested by bioluminometry, and compared with the biomass viability in the free form ***(9,12)***. Determination of yeast biomass immobilized in a range of ionotropic hydrogels was also published, and as an example, this method is discussed in the experimental section in detail.

1.4. Bioluminescence-Based Biosensors

Biosensor techniques based on bioluminescence, and also on chemiluminescence, have become very popular in the recent period mainly as a result of the advances in light detection. Light detection with the latest models of photomultipliers and charge-coupled device (CCD) cameras is very efficient and actual instruments are small and portable as well as relatively affordable compared to other techniques. Luminometry has some advantages over other optical techniques: extraordinary sensitivity and wide dynamic range. Luminometry is up to 10^5 times more sensitive than absorption spectrometry. Theoretically speaking, because single photon can be detected in photomultiplier a single molecule of analyte or a single cell can be detected. In real systems however light detection is not that efficient but still bioluminescent and chemiluminescent techniques can detect trace amounts of analytes or cells.

The major field of application of bioluminescence biosensors, exploiting immobilized intact cells, is environment monitoring. Applications of luminescence biosensors in the environment monitoring were extensively reviewed ***(26,27)***. Luminescence-based biosensor techniques have been extensively used for monitoring of various organic and inorganic chemical contaminants. Various toxic compounds and environmental contaminants can generally interfere with energetic metabolism and electron transfer system. These produce substrates for biolumi-

Table 1
Bioluminescence-Based Microbial Biosensors

Analyte/Field of Application	Light generation microorganism	Ref.
Hg^{2+}, heavy metals	*Vibrio fischeri* (gen. modified)	***13***
Zn^{2+}, heavy metals	Cyanobacteria	***14***
Heavy metals, pentachlorophenol	*Photobacetrium phosphoreum*	***15***
β-Lactam antibiotics	*Escherichia coli* (gen. modified)	***16***
Polycyclic aromatic hydrocarbons (PAH)	Gen. modified bacteria	***17***
PAH	recombinant *E. coli*	***18***
Genotoxic agents	*Escherichia coli*	***19***
Heavy metals	*Alcaligenes eutrophus*	***20***
Herbicides	Cyanobacteria	***21***
Phenolics	recombinant *E. coli*	***22***
Hydrogen peroxide, phenol, mitomycin C	*E. coli*	***23***
Naphthalene	*Pseudomonas putida*	***24***
Benzene, toluene, ethylbenzene, xylene	Recombinant bioluminescent bacterium	***25***

nescence and therefore any interference results in effects observable as a loss of luminescence. Recently published applications of the luminescence biosensor exploiting immobilized cells are summarized in **Table 1**.

Because the entire metabolism involved in bioluminescence is intracellular, effects on bioluminescence are likely to occur as a response to toxic compounds available in a form, which can be transported into the cell. As a result of the transport processes, correlation between concentrations of active compound is not always satisfactory and is usually very specific to each microorganism-toxic compound system.

Immobilization techniques used to immobilize bioluminescent cells are in general any gentle immobilization techniques used in other fields that can preserve the viability of the immobilized cells. Another crucial requirement is optical property of an immobilization material resulting in minimal attenuation of bioluminescence. The use of several immobilization materials and techniques was reported: alginate ***(19)***, agar ***(25)***, porous sol-gel glass ***(28)***, immobilization on a gold surface ***(29)***, and several reports of immobilization on polyamide membranes ***(30–32)***.

Because of their relative simplicity and capability to detect contaminants dangerous to humans, bioluminescence biosensors have great potential in the environment monitoring. Main advantages that make bioluminescence methods so attractive are: extreme sensitivity and speed compared to other methods, ease of implementation, and potential to be used in portable field devices.

2. Materials

2.1. Instrumentation

1. Bio-Orbit 1253 Luminometer (Bio-Orbit, Turku, Finland) equipped with a recorder or a computer for data collection.
2. High-precision analytical balance.

2.2. Reagents (see Notes 1 and 2)

1. Monitoring kit (1243-114) containing following reagents, delivered by Bio-Orbit.
2. 1243-251 Saline: 0.9% w/v NaCl in double distilled water, sterile.
3. 1243-244 Diluent: 100 m*M* Tris-acetate buffer, 2 m*M* ethylene diamine tetraacetic acid (EDTA), pH 7.75 at 25°C, sterile.
4. 1243-250 ATP Releasing Buffer (ionic surfactant for releasing biological ATP).
5. 1243-209 ATP Monitoring Reagent (firefly luciferase, D-luciferin, bovine serum albumin, magnesium acetate, inorganic pyrophosphate).
6. 1243-118 ATP Standard, if ATP content is to be measured.

2.3. Bioluminometric Assay

1. Transparent cylinder-shaped 4 mL polystyrene cuvets, disposable (*see* **Note 3**).
2. Micropipets with disposable tips (volumes 10–500 µL, *see* **Note 3**).

3. Methods

3.1. Bioluminometric Biomass Determination

1. Weigh an empty measuring cuvet.
2. Take about 5 to 10 gel beads with a 2- to 3-mm diameter (or an equivalent corresponding to 1 to 80 mg of dry cell weight), taken either immediately after immobilization or removed from a cultivation flask, and dry them quickly with a piece of blotting paper (*see* **Note 4**).
3. Place them into the cuvet and weigh again.
4. Add 500 µL ATP releasing buffer (ATP-RB) to the cuvet to extract intracellular ATP from cells.
5. Mix by modest spinning for about 20 s.
6. Insert the cuvet into the measuring chamber of the luminometer and record background light emission (baseline).
7. Add 500 µL of ATP monitoring reagent (ATP-MR), mix quickly, and place the cuvet back into the measurement chamber and measure light output. The light output (LO) is given in relative light units (RLU), and the response corresponds directly to the ATP level present in the sample (*see* **Fig. 1**).

3.2. Evaluation of Biomass Concentration

1. Calibration of the method must be performed for every cell type separately. 250 µL of standards containing between 1 and 80 mg 100% active cell biomass suspended in 1 mL of re-distilled water, are measured by luminometer, followed the procedure given in **Subheading 3.1.** Calibration line showing the relation between the response and the biomass concentration in a standard is then constructed.

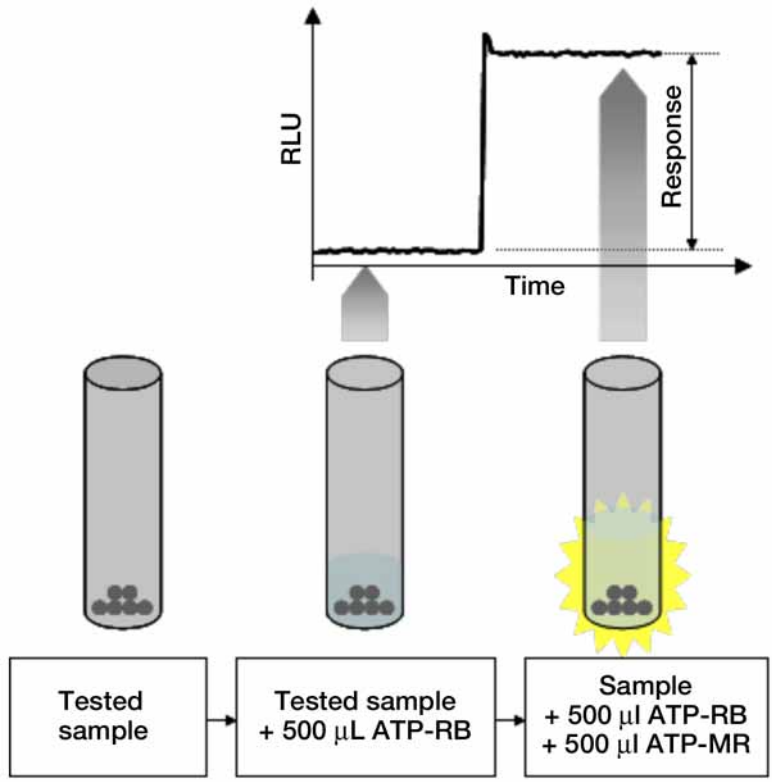

Fig. 1. Scheme for measurement and evaluation of extracelullar ATP for active biomass detection.

2. Biomass concentration in test samples is calculated by using response (R) in RLU values (the baseline signal is subtracted from that measured upon addition of the ATP monitoring reagent). The formula to calculate the biomass concentration in samples containing immobilized cells is as follows:

$$\text{Biomass conc}\left[\frac{g_{DCW}}{g}\right] = \frac{S\left[\frac{mg}{mL}\right] \times 0.25\ mL}{m[g]} \quad (2)$$

where S is standard concentration in mg/mL equivalent to the Response value of the sample (based on the calibration line) and m is weight of the gel beads in grams (g).

3.3. Calculation of Adenosine Triphosphate Content

1. Samples can be analyzed for concentration of intracellular ATP using the ATP standard contained in the kit. *Note:* The ATP standard must be further diluted before use! See instructions for more information!

2. Analysis, as described in the **Subheading 3.1.** is performed, and followed by addition of the ATP standard for internal calibration.
3. After copmleting **Subheading 3.1.**, **step 7** add 10 µL reconstituted ATP standard (10^{-6} *M*), mix quickly, place the cuvet back into the measurement chamber and measure light output. Increased response (*IR*) is given in RLU, which corresponds directly to the ATP level present in the sample and the ATP standard.
4. ATP content in test is calculated by using RLU values of the baseline subtracted from those measured upon addition of the ATP monitoring reagent (*R*) and those measured upon addition of the ATP standard (*IR*). The formula to calculate the biomass concentration is samples containing immobilized cells is as follows:

$$\text{ATP cont.}\left[\frac{\mu\text{g}}{\text{g}}\right] = \frac{R[\text{RLU}] \times c_s\left[\frac{\text{mol}}{\text{L}}\right]}{\left(IR[\text{RLU}] - \frac{R[\text{RLU}]}{m[\text{g}]}\right)} \quad (3)$$

where *R* is a response value of the sample in RLU; *I*R is an increased response value of the sample + ATP standard in RLU; c_s is the ATP standard concentration (10^{-6} mol/L for the ATP standard contained in the kit) and *m* is weight of the gel beads in grams (g).

4. Notes

1. Reagents for bioluminometric analysis must be reconstituted prior to the analysis. Follow kit instructions on how to reconstitute the reagents contained in the kit.
2. Reagents other than those delivered by Bio-Orbit may also be used. We have obtained good results using Microbial Biomass Kit (Celsis, Newmarket, UK).
3. Disposable cuvettes and pipette tips are advised to use to avoid introducing foreign nucleotides into the sample.
4. The amount of immobilized cells used may differ according to the type of cells used. Estimation of the span may be required prior to the analysis.

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35

A Proteomic Approach to Biofilm Cell Physiology

Laurent Coquet, Sébastien Vilain, Pascal Cosette, Thierry Jouenne, and Guy-Alain Junter

Summary

Proteomic analyses are increasingly implemented to investigate the particular physiology of (naturally or artificially) immobilized microorganisms. The protein maps of immobilized cells are compared with those of suspended counterparts to reveal alterations in protein expression induced by the microbial mode of growth. Proteins whose amount significantly varies between free and immobilized cells are identified by mass spectrometry and referring the peptide fingerprints to published databases. This proteomic approach is illustrated here using *Pseudomonas aeruginosa* cells grown as biofilms on clay beads for 24 or 48 h. Both the growth mode (suspended or attached) and the incubation time were shown to control the expression level of a large number of proteins by *P. aeruginosa*. Proteins whose amount significantly varied in biofilm organisms compared to suspended bacteria could be divided into three main classes, namely proteins linked to metabolic processes, proteins involved in adaptation and protection, and membrane proteins.

Key Words: Biofilm; 2D-electrophoresis; immobilized cells; *P. aeruginosa*; proteome.

1. Introduction

Microbiologists have shown a great interest for the sessile microbial growth mode over the past two decades. Indeed, it is now admitted that in most ecosystems, microorganisms predominate as surface-attached and matrix-enclosed communities called biofilms ***(1)***. Despite this definite importance of the sessile state in microbial way of life and its consequences for human beings, our present knowledge of the physiology of sessile bacteria remains still fragmentary. Thus, the mechanisms involved in the resistance of biofilm cells to antimicrobials (one of the main characteristic of biofilm cells) are complex and still not fully understood ***(2)***.

The publication of the first complete genome sequence of a living organism in 1995 opened a new era in biology ***(3)***. The exponential increase in genome sequence information that followed has prompted researchers to design new "global" experiments dealing with functional genomics. Transcriptomics, relying on DNA

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arrays, provides information on the global gene expression pattern of a cell. Proteome—defined by Wilkins as all proteins expressed by a genome, cell or tissue ***(4)***—provides additional information on the amount, modification and subcellular localization of molecules that act as cellular effectors (i.e., proteins). Geneticists, molecular biologists, and biochemists have logically applied these new tools to biofilms ***(5)***. However, all these data need an excellent experimental design. The present chapter gives appropriate methods for assessing alterations induced by cell adhesion on bacterial proteome. As an example, sessile cells are obtained by colonizing clay beads ***(6)*** but the procedure applies as well to microbial cells recovered from other model biofilms ***(7)***.

2. Materials

2.1. Cultivation of Planktonic and Sessile Bacteria (see Note 1)

1. Clay beads.
2. Minimal salt medium, pH 7.5: 0.6 g/L Tris-base; 14.97 g/L Tris-HCl, 0.5 g/L NH_4Cl, 0.05 g/L $CaCl_2$, 0.05 g/L $MgSO_4$, $7H_2O$, 0.005 g/L $FeSO_4$, $7H_2O$, 0.005 g/L $MnSO_4$, H_2O, 15 g/L glucose, 2 g/L yeast extract.
3. 250-mL Erlenmeyer flasks.
4. Glass column with a recycle loop (*see* **Note 2**).
5. 1-L Glass tank.

2.2. Recovery of Bacteria

1. 0.1 *M* Potassium phosphate buffer, pH 7.0.
2. Sterile flasks.
3. Glass beads (3-mm diameter).
4. Glass-fiber membrane (GF/C Whatman).

2.3. Preparation of Crude Protein Extracts

1. 0.1 *M* phosphate buffer, pH 7.0.
2. IEF buffer: 5 *M* urea, 2 *M* thiourea, 2% (w/v) 3-[3-cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS), 2 m*M* tributyl phosphine (*see* **Note 3**), 10 m*M* dithiothreitol (DTT), and 2% (v/v) carrier ampholytes, pH 3.5–10 (Amersham).
3. Bio-Rad protein assay kit.

2.4. Rehydratation of IPG Strips

1. IEF buffer.
2. Coomassie Brilliant Blue R-250 (Sigma).
3. Reswelling cassette (Amersham Biosciences).
4. Mineral oil (Sigma).

2.5. First Dimension: Isoelectric Focalization

1. Immobilized pH gradient (IPG) Strips (Immobiline Dry Strip 18 cm, pH 3.0 to 10.0, nonlinear).

2. Multiphor II horizontal electrophoresis apparatus (Amersham Biosciences), power supply (3500 V), thermostatation by tap water circulation, IEF sample applicator strip (Amersham Biosciences).
3. IEF electrode strips.
4. Mineral oil.
5. Deionized water.
6. Micropipet.
7. Clip.
8. Gloves.
9. Plastic film.

2.6. IPG Strip Equilibration

1. Equilibration buffer No. 1: 6 *M* urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecylsulfate (SDS) and 2% (w/v) DTT in 50 m*M* Tris-HCl, pH 6.8.
2. Equilibration buffer No. 2: 6 *M* urea, 30% (v/v) glycerol, 2% (w/v) SDS, 2.5% (w/v) iodoacetamide and 0.03% (w/v) Commassie Brilliant Blue R-250 (Sigma) in 50 m*M* Tris-HCl, pH 6.8.

2.7. Second Dimension: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

1. Protean II xi cell electrophoresis apparatus (BioRad).
2. Polyacrylamide solution: 40 g polyacrylamide + 100 mL water
3. 2% (w/v) Bis-acrylamide solution.
4. 20% (w/v) SDS solution.
5. Tetramethyleneethylenediamine (TEMED) solution (Sigma).
6. 10 % (w/v) Ammonium persulfate solution.
7. Stacking gel buffer:1 *M* Tris-HCl, pH 6.8.
8. Resolving gel buffer: 1 *M* Tris-HCl, pH 8.8.
9. Electrode buffer solution: 3 g/L Trizma base, 14 g/L glycine, 1 g/L SDS in deionized water.
10. Vacuum flask.
11. Spatula.

2.8. Gel Silver Staining

1. Fixation solution: 10% (v/v) acetic acid and 30% (v/v) ethanol in deionized water.
2. 10% (v/v) Ethanol solution.
3. 0.02 % (w/v) Sodium thiosulfate solution in deionized water.
4. 0.1 g/L Silver nitrate solution in deionized water.
5. Development solution: 24 g sodium carbonate, 200 µL of 37% formaldehyde solution and 80 µL of 2% sodium thiosulfate solution in 2 L deionized water.
6. 1% (v/v) Acetic acid solution.
7. 3% (v/v) Glycerol solution.

2.9. Gel Analysis

1. Gel scanning densitometer (e.g., Bio-Rad GS 800).
2. Gel analysis software (PDQuest software [Bio-Rad] or Melanie 4 (Amersham Biosciences).

2.10. Enzymatic Digestion of Proteins

1. Sequencing grade modified trypsin (Promega, Madison, WI).
2. Sterile scalpel.
3. Acetonitrile (CH_3CN).
4. 10 m*M* Ammonium bicarbonate solution.
5. Extraction solution: water/CH_3CN/trifluoroacetic acid (TFA) (79/20/1).
6. 5% (v/v) Formic acid solution.

2.11. MALDI-TOF Analyses

1. The matrix is constituted of 3 mg/mL α-cyano-4-hydroxycinnamic acid diluted in a 60 % (v/v) CH_3CN and 0.1 % (v/v) TFA solution.
2. Dessicator.
3. Mass spectrophotometer (proTOF™ 2000, Perkin Elmer Sciex, Boston, MA).

3. Methods

3.1. Cultivation of Biofilm Cells

1. Put sterile clay beads into the flow cell (*see* **Note 4**).
2. Fill the glass tank with 800 mL of minimal salt medium and connect it with the recycle loop.
3. Use a peristaltic pump to apply a recycle flow rate of 0.7 mL/min.
4. Put the tank in a water bath maintained at 37°C.
5. Run the reactor for 18 or 48 h.

3.2. Cultivation of Planktonic Cells

Apply the same protocol than for biofilm cells but in the absence of clay beads.

3.3. Recovery of Bacteria

1. Remove aseptically (*see* **Note 4**) the clay beads.
2. Wash the beads twice with 80 mL of 0.1 *M* potassium phosphate buffer, pH 7.0 in a 250-mL Erlenmeyer flask.
3. Place the beads into a sterile flask containing 30 g of glass beads and 50 mL of potassium phosphate buffer, pH 7.0.
4. Shake vigorously the beads for 20 min.
5. Filter twice the bacteria suspension under vacuum.
6. Centrifuge the suspension at 1500*g* for 15 min.
7. Resuspend the bacterial pellet in 5 mL 0.1 *M* phosphate buffer, pH 7.0.

3.4. Preparation of Crude Protein Extracts

1. Centrifuge the bacterial suspension at 1500*g* for 15 min.
2. Wash the pellet in 0.1 *M* phosphate buffer, pH 7.0, and pellet cells at 1500*g* for 15 min.
3. Resuspend the pellet in IEF buffer (about 0.2 g wet weight pellet per 2 mL buffer).
4. Use latex gloves from this step.
5. Disrupt cells by thermal shock (from –24°C to 20°C) and then by ultrasonication (30 W; 15 pulses of 2 s separated by 2-s breaks). This last procedure must be performed at 4°C.

6. Centrifuge the protein extract at 10,000*g* for 20 min to eliminate cell debris.
7. Protein amounts in the supernatant are evaluated using the Bio-Rad protein assay by measuring the absorbance at 595 nm (*see* **Note 5**).
8. Store the supernatant at –80°C.

3.5. Rehydration of IPG Strips

1. Calculate the sample volume necessary to obtained 100 µg of proteins.
2. Transfer this sample to a 1.5-mL-Eppendorf tube and complete to 400 µL with IEF buffer containing 1% (m/v) Coomassie Brilliant Blue R-250 (*see* **Note 6**).
3. Freeze the sample at –24°C.
4. Fill each immobiline drystrip reswelling tray with a protein sample (*see* **Note 7**).
5. Spread the protein sample with a spatula.
6. Pull off the protective covers of the IPG strips.
7. Put the IPG strips carefully into the rehydration cassette, the gel slide directed towards the protein sample (*see* **Note 8**).
8. Cover the IPG strips with mineral oil.
9. Rehydrate the strips overnight at room temperature.

3.6. Isoelectric Focalization

1. Moisten the cooling plate of the Multiphor II apparatus with 20 mL of mineral oil.
2. Place the tray and electrode holder onto the cooling plate.
3. Pour 15 mL of mineral oil into the tray.
4. Place the Drystrip aligners (*see* **Note 9**).
5. Remove the IPG strips from the rehydratation cassette by using a clip.
6. Place the IPG strips on the DryStrip aligners, their acidic end towards the anode.
7. Cut two IEF electrode strips to a length corresponding to the width of the IPG strips.
8. Soak the electrode strips with deionized water.
9. Place the IEF electrode strips on top of the aligned IPG gel strips at the cathodic and anodic ends.
10. Position the electrodes and press them gently down on top of the IEF electrode strips.
11. Cover the IPG strips with mineral oil.
12. Place the lid, connect the cables to the power supply.
13. Maintain the temperature of the tray by a circulation of tap water.
14. Start the IEF. Running conditions depend on the pH gradient and the length of the IPG strips used. For a pH of 3.0 to 10.0 and a strip of 18 cm, the time schedule is the following: 150 V for 1 h, 350 V for 15 min, 750 V for 45 min, 1.5 kV for 1 h and 3.5 kV for 17 h (1 mA, constant) for a total of 61.8 kVh.
15. After IEF, store the IPG strips between two sheets of plastic film at –24°C.

3.7. Equilibration of IPG Gel Strips

1. Defrost the IPG strips.
2. Equilibrate successively the IPG strips in 15 mL of each equilibration buffer for 10 min each time under slight agitation.
3. Blot the IPG strips to remove excess equilibration buffer.

3.8. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (see Note 10)

1. Transfer in vacuum flask (for 1 gel) 15.70 mL of 40% polyacrylamide solution, 8.39 mL of 2% *bis*-acrylamide solution, 19.35 mL of Tris-buffer, pH 8.9, and 7.70 mL of deionized water (*see* **Note 11**).
2. Remove air under vacuum.
3. Add 258 µL of 20% SDS solution, 40.50 mL of TEMED and 154.8 µL of 10 % ammonium persulfate solution.
4. Cast immediately the running gel by using a casting stand of the Protean II xi Cell apparatus (gel size 200 × 250 × 1 mm^3). Avoid introduction of any air bubbles.
5. Carefully pipette 2 mL of overlay propan-2-ol 50% in water onto the top of the gel.
6. Allow the gel to polymerize for 45–60 min at room temperature.
7. Remove propan-2-ol and add water from the top of the gel for the night.
8. Remove water.
9. Transfer in vacuum flask 923 µL of 40% polyacrylamide solution, 491 µL of 2% *bis*-acrylamide solution, 1.01 mL of 1 *M* Tris-buffer, pH 6.8, and 5.59 mL of water (*see* **Note 11**).
10. Remove air under vacuum.
11. Add 40.6 µL of 20% sodium dodecyl sulfate (SDS) solution, 26.5 µL of TEMED and 40.6 µL of 10% ammonium persulfate solution.
12. Cast immediately the stacking gel onto the top of the running gel.
13. Add propan-2-ol 50% on the top of the gel.
14. Allow the gel to polymerize for 45 to 60 min at room temperature.
15. Remove propan-2-ol and rinse with distilled water.
16. Place each IPG strip on top of the stacking gel. Carefully press the IPG strip with a spatula onto the surface of the gel.
17. Fill the buffer tank of the electrophoresis apparatus with the electrode buffer.
18. Insert the gel cassette in the electrophoresis apparatus. Thermostate at 8°C with polyethyleneglycol.
19. Put the lid on the electrophoresis unit and connect cables.
20. Start SDS PAGE at 20 mA/gel for about 1 h with a limit of 150 V.
21. Put 40 mA/gel and a limit of 350 V.
22. When Coomassie Brilliant Blue tracking dye has migrated off the lower end of the gel, terminate the run.
23. Remove the gel from the buffer tank.
24. Peel carefully the gel from the glass plate and remove the IPG strip and the stacking gel with a spatula.

3.9. Gel Staining

1. Fix the gel from 3 h to overnight in the fixation solution.
2. Rinse twice the gel in the 10% ethanol solution for 10 min.
3. Rinse 3 × 10 min in deionized water.
4. Soak gel for 1 min in 0.02 % sodium thiosulfate solution.
5. Rinse for 1 min in deionized water.
6. Impregnate for 30 min in silver nitrate solution.

7. Rinse in water for 30 s.
8. Develop image for 20 to 30 min in the development solution.
9. Stop development in 1% acetic acid solution during 10 min.
10. Soak in a 3% glycerol solution.

3.10. Gel Analysis

1. Scan the gel using a densitometer.
2. Analyze the gel using a software (PDQuest or Melanie 4).

3.11. Protein Identification

1. Excise the spots from the gel with a sterile scalpel and slice into small pieces.
2. Wash gel plugs twice for 15 min with 100 µL of deionized water.
3. Wash plugs twice with 100 µL of H_2O/CH_3CN (1/1 v/v) for 15 min.
4. Place plugs in 20 µL of 100% CH_3CN for 15 min.
5. Dry plugs using a SpeedVac centrifuge for a few minutes.
6. Add 15 µL of a 20 ng/µL trypsin solution.
7. After rehydratation (30 min), cover the plugs with 20 µL of 10 m*M* ammonium bicarbonate solution and after 3 h, add 35 µL of water.
8. Allow digestion overnight at 37°C.
9. Collect the liquid phase containing peptides.
10. Add 20 µL of H_2O/CH_3CN/TFA mixture (79/20/1) to remove peptides remaining in the gel.
11. Pull the different fractions.
12. Dry in a vacuum centrifuge.
13. Redissolve in 10 µL of 5% formic acid.
14. 1 µL Peptide solution is mixed with 1 µL of the matrix solution.
15. 1 µL Mixed peptide-matrix is deposed on the target-plate.

The peptide fingerprints can be matched against *in silico* digests using the MS-FIT software with the GenePept database restricted to *Pseudomonas aeruginosa*, accessible at http:\\prospector.ucsf.edu.

3.12. A Practical Example: Standard Proteomic Maps for Planktonic and Clay-Bead-Attached P. aeruginosa *Cells*

1. **Figure 1** shows standard proteomic maps for planktonic (F) and clay-bead-attached (CB) *P. aeruginosa* cells after incubation for 18 or 48 h.
2. A total of 886 protein spots were discriminated on 2-DE electropherograms and quantified by computing scanning densitometry.
3. The corresponding proteins were expressed in at least one of the 4 tested incubation conditions (i.e., they were present in at least one of the four synthetic gels created by the PDQuest software).
4. The proteins produced by attached bacteria could be divided into three families by comparing their spot intensities to those of spots from suspended-cell electropherograms (i.e., underproduced, overproduced, and unaffected peptides). The levels of a large number of proteins differed between biofilm organisms and suspended bacteria (**Table 1**). These differences depended both on the growth mode (suspended or attached) and the incubation time.

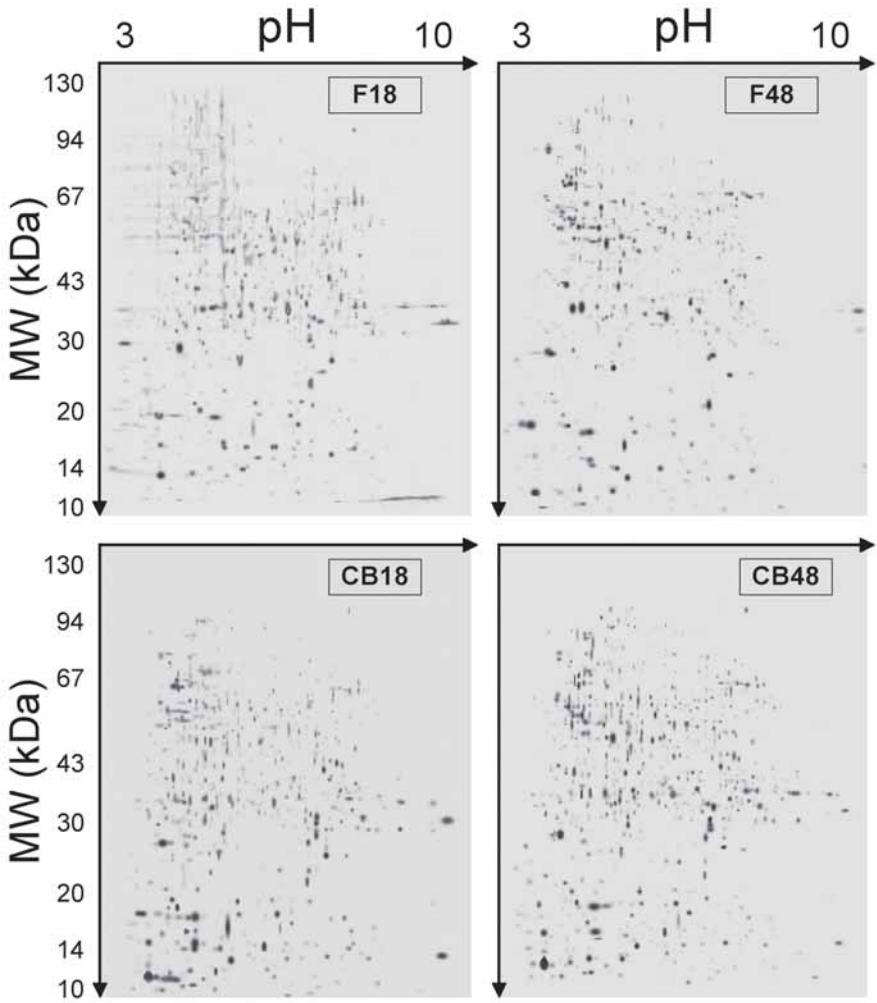

Fig. 1. Standard proteomic maps for planktonic (F) and clay-bead-attached (CB) *P. aeruginosa* cells after incubation for 18 or 48 h. Each standard proteomic map was obtained from three experimental 2D gels. Images were analyzed by using the PDQuest software (version 6.21).

5. A number of proteins whose amount significantly varied in biofilm organisms compared to suspended bacteria were identified (**Table 2**).
6. These proteins can be divided into three main classes. The first class includes proteins linked to metabolic processes, in particular enzymes, showing (not surprisingly) that central metabolism is altered by the sessile mode of growth. The second class includes proteins involved in adaptation and protection. This general stress response initiated by growth within a biofilm might explain the resis-

Table 1
Number of Proteins Whose Amount Significantly Varied in *P. aeruginosa* Cells According to the Incubation Conditions.

	Number of spots						
X/Y[a]	Total	Absent in X and Y	Absent in X	Absent in Y	+[b]	–	=
CB18/F18	816	70	202	113	48	130	638 (78.2%)
CB48/F48	842	44	114	170	63	78	701 (83.3%)
F48/F18	782	104	110	79	59	59	664 (84.9%)
CB48/CB18	789	97	62	175	69	25	695 (88.1%)

*Spots displaying statistically different protein quantities in organisms recovered from X cultures compared to Y cultures fulfilled the following criteria: *p*-values of ≤ 0.05 (*t*-test); fold change ≥ 2; average volume ≥ 20 ($n = 3$).

[a]Incubation conditions X and Y were compared.

[b]+, overproduced in X; – underproduced in X ; =, no difference

Abbr: CB18, attachment to clay beads for 18 h; CB48, attachment to clay beads for 48 h; F18, control free-cell cultures incubated for 18 h; F48, control free-cell cultures incubated for 48 h.

tance of sessile cells to environmental stresses ***(8)***. The third one is composed of membrane proteins. Membrane proteins have been reported to have a substantial influence on attachment and may also play a role in early biofilm development ***(9,10)***. They are implied in multidrug resistance pumps of gram-negative bacteria ***(11,12)*** and their over/underproduction by ICs may therefore be implied in IC resistance to antibiotics. The expression level of some proteins involved in DNA transcription and translation was also affected by immobilization. So, many cellular functions were altered by the sessile mode of growth, which confirms a number of literature data ***(13–16)***. In addition, the biofilm mode of growth also affected a great number of proteins with an unknown function.

8. Here, most identified proteins, whatever their function, were underproduced by biofilm cells compared to control, suspended counterparts. However, no clear expression tendency of proteins whose level was affected in sessile bacteria could be discerned when extending the proteomic analyses to other immobilized-cell systems, either natural (biofilm formation on glass wool fibres) or artificial (entrapment in agar gel layer) ***(17)***. Furthermore, the discrimination between trivial and key peptides (i.e., those whose variations in expression level will exert deciding influence on the physiology of immobilized cells) among those differentially expressed by sessile bacteria as compared to free counterparts seems a difficult challenge. It may also be difficult (and sometimes dangerous) to advance a specific role for a given over/underexpressed protein in the biofilm phenotype.
9. In conclusion, the emergence of proteomics as a powerful tool to compare the global regulation patterns of gene expression in free and immobilized microbial cells opens promising avenues to the study of immobilized cell physiology. The

Table 2
Identification and Function of Proteins Underproduced or Overproduced in Biofilm Cells Compared to Suspended Counterparts

Function Protein (gene)	Estimated *pI*	Estimated MW (kDa)	Locus[a]	Level[b]
Metabolism				
Serine protease MucD precursor (*mucD*)	7.38	58	PA0766	–[18]
Phenylalanine-4-hydroxylase (*phhA*)	7.23	16	PA0872	–[18]
Acetyl-CoA acetyltransferase (*atoB*)	7.36	48	PA2001	–[18]
NADH Dehydrogenase I chain M (*nuoM*)	7.30	30	PA2648	–[18]
3-Oxoacyl-[acyl-carrier-protein] reductase (*fabG*)	7.61	26	PA2967	–[18]
Probable peroxidase	5.01	26	PA3529	–[48]
3-Hydroxyisobutyrate dehydrogenase (*mmsB*)	7.16	31	PA3569	–[18]
Probable iron-sulfur protein	6.62	24	PA4431	+[18,48]
Azurin precursor (*azu*)	6.06	16	PA4922	–[18]
Acetolactate synthase isozyme III small subunit (*ilvH*)	7.21	16	PA4695	–[18]
Nitrogen regulatory protein P-II 2 (*glnK*)	5.01	14	PA5288	–[48]
Orotate phosphoribosyltransferase	4.66	28	PA5331	+[48]
Transcription/Translation				
50S Ribosomal protein L10 (*rplJ*)	9.29	16	PA4272	+[18,48]
Probable ribosomal protein L25	7.25	27	PA4671	–[18]
Adaptation/Protection				
Bacterioferritin comigratory protein (*bcp*)	8.39	15	PA1008	–[48]
Pyocin S2 immunity protein (*imm2*)	8.39	12	PA1151	–[48]
Probable cold-shock protein	8.50	12	PA1159	+[48]
Heat-shock protein IbpA (*ibpA*)	6.77	15	PA3126	–[48]
Superoxide dismutase (*sodB*)	4.69	20	PA4366	–[18]
Membrane Proteins/Transport/Binding Proteins/Motility				
Branched-chain amino acid transport protein BraC (*braC*)	4.53	52	PA1074	–[18]
Flagellin type B (*fliC*)	4.30	55	PA1092	–[18]
Binding protein of ABC phosphonate transporter	6.88	37	PA3383	–[18]
Hypothetical				
Conserved hypothetical protein	4.60	18	PA0083	+[18]
Hypothetical protein	8.18	17	PA0315	–[48]
Hypothetical protein	7.10	28	PA0943	–[18,48]

continued

Table 2 (Continued)

Function Protein (gene)	Estimated *pI*	MW (kDa)	Locus[a]	Level[b]
Hypothetical protein	7.61	16	PA1746	$-^{18}$
Hypothetical protein	7.25	19	PA2575	$-^{18,48}$
Hypothetical protein	8.78	29	PA3313	$+^{18,48}$
Conserved hypothetical protein	8.23	29	PA3731	$+^{48}$
Conserved hypothetical protein	7.08	36	PA4352	$-^{18,48}$
Conserved hypothetical protein	9.18	19	PA4453	$+^{18}$
Conserved hypothetical protein	5.71	15	PA4463	$-^{18,48}$
Hypothetical protein	5.13	30	PA4495	$-^{48}$
Conserved hypothetical protein	8.09	15	PA4739	$-^{48}$
Conserved hypothetical protein	5.03	15	PA5178	$-^{18,48}$

Notes:[a]Protein identification number according to *Pseudomonas* genome project database (http://www.pseudomonas.com).

[b]+, overexpressed; –, underexpressed; superscript, duration of incubation.

alliance of the proteomic approach with classical tools of molecular biology (e.g., mutagenesis aapproaches) will probably allow in the near future to propose new targets against harmful biofilms (e.g., by identifying key proteins such as genetic determinants of biofilm resistance to antimicrobials).

4. Notes

1. All materials must be sterilized at 120°C during 15 min before use.
2. The device has been described in the paper by Vilain et al. ***(6)*.**
3. Caution, explosive after contact with air, work in a chemical hood.
4. Experiments should be performed in a bacteriological hood.
5. A calibration curve must be built by using increasing concentrations of bovine serum albumin (Sigma).
6. Better results are obtained by substituting Bromophenol Blue with Coomassie Brilliant Blue ***(6)***.
7. Fill from the top with a pipet.
8. Use a micropipet and eliminate air bubbles.
9. Avoid air bubbles.
10. The gel must be made the previous day.
11. The solutions must be precooled at 4°C.

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Encapsulation of Bacteria for Biodegradation of Gasoline Hydrocarbons

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Summary

Gasoline hydrocarbons are a common source of contamination to soil and aquifer systems. Biodegradation of these contaminants by hydrogel-encapsulated bacteria is a novel technique for bioremediation of contaminated sites. Hydrogel capsules provide a stable, consistent, and protective microenvironment for prolonged survival and metabolic activity of encapsulated cells. This monograph presents the materials and methods for encapsulation of gasoline-degrading bacteria in gellan gum microspheres. The microencapsulation process is based on a two-phase dispersion technique, termed emulsification-internal gelation. The sphere formation process involves the dispersion of two immiscible liquid phases resulting in a water-in-oil (w/o) emulsion. A suspension of viable cells in an aqueous solution of gellan gum (disperse phase) is emulsified in a hydrophobic phase such as canola oil (continuous phase). The gelation of the small droplets of the dispersed phase is subsequently initiated by decreasing the emulsion temperature. Changing the emulsion conditions can alter the size distribution of microspheres. The emulsification-internal gelation method is applicable to encapsulation of other microbial degraders in macro- or microspheres subject to the employment of proper emulsion conditions.

Key Words: Biodegradation; gasoline hydrocarbons; bacteria; encapsulation; emulsification-internal gelation; gellan gum.

1. Introduction

The release of petroleum products such as gasoline from leaking underground storage tanks is a common source of contamination to soil and groundwater ***(1–3)***. Gasoline is a light distillate and may contain C_4 to C_{11} alkane, cycloalkane, alkene, and aromatic hydrocarbons ***(4)***. These compounds are sufficiently soluble in water to pose a major treat to groundwater quality. Aromatic components such as benzene, toluene, ethylbenzene and xylenes (ortho-, meta-, and para-xylene), collectively referred to as BTEX, comprise between 20% and 30% of commercial

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gasoline. The BTEX compounds are typically the targets of regulatory concern and the United States Environmental Protection Agency has classified them as hazardous waste ***(5)***. The BTEX compounds are relatively water-soluble and mobile, and therefore, even a small discharge of gasoline has the potential to render large portions of groundwater unfit for drinking.

Gasoline hydrocarbon components may be transferred from the fuel liquid phase to the air, water, and soil phases by a variety of physical, chemical, and biological processes that can occur in the subsurface. Upon the release of gasoline into a subsurface environment, a minor fraction of gasoline hydrocarbons may be subject to evaporative and photodegradative losses. However, the majority of gasoline hydrocarbons will be subject to vertical and horizontal infiltration at a rate determined primarily by the stratification and permeability of the soil materials. Infiltrated hydrocarbons migrate by gravity downward through unsaturated soil layers (vadose zone) and may eventually reach the soil water table (capillary zone) where they form a floating light nonaqueous phase liquid (LNAPL). A small portion of hydrocarbons is dissolved in groundwater (saturated zone), forming a contaminant plume, which spreads within the direction of bulk groundwater flow. Residual floating hydrocarbons may spread out laterally and act as a long-term source of groundwater contamination.

It has been known since the 1940s that some microorganisms are capable of degrading petroleum hydrocarbons ***(6)***. However, it was only after the accidental release of massive volumes of gasoline and crude oil into aquatic environments in the late 1960s ***(7)*** and the early 1970s ***(8)*** that biodegradation was actively considered as a remedial strategy to clean up contaminated groundwater and marine systems.

The biodegradation of gasoline hydrocarbons as the common environmental pollutants has been widely studied in the laboratory ***(9–17)*** and field studies ***(18–22)***. Aromatic components of gasoline (BTEX) have also been the main focus of several biodegradation studies ***(23–29)***. The microbial degradation of hydrocarbons is strongly influenced by physical and chemical factors such as temperature, oxygen, nutrients, salinity, water activity, pH, and concentration and availability of the contaminant, and also by biological factors such as the composition, adaptability, and physiological capabilities of the microbial populations.

Over the years, a wide variety of engineered *in situ* and *ex situ* bioremediation technologies have been developed ***(5,30)***. These technologies are mainly designed to increase the availability of electron acceptors (oxygen or nitrate), electron donors (organic substrates), nutrients (nitrogen, phosphorous, or potassium), or degrading microbial communities. The last approach is generally referred to as bioaugmentation, which is defined as the process of adding specifically adapted exogenous microorganisms to a system to stimulate biodegradation and enhance the rate and/or extent of biodegradation.

Bioaugmentation of subsurface polluted environments (soil and groundwater) or surface bioreactors with pre-isolated microorganisms capable to degrade certain pollutants is an alternate remedy for decontamination of sites or industrial waste effluents ***(31,32)***. Successful application of living microorganisms to bioaugmentation schemes depends on inoculum density and formulation, mode of application (single or multiple introductions), mode of operation, biotic and abi-

Table 1
Characteristics of Microbial Supports for Environmental Applications

Shape
Size (surface area-to-volume ratio)
Porosity
Toxicity
Biocompatibility
Cell mass loading capacity
Retention capacity
Performance quality
Stability (chemical, biological, mechanical, and thermal)
Ease of production
Reusability
Longevity
Cost (economic feasibility)

otic environmental effects, rate of survival, and the extent of distribution and transport of added microorganisms (e.g., through the soil matrix). Both biotic and abiotic factors play critical roles in determining the survival of introduced microorganisms. In subsurface applications, biological factors include predation by protozoans, lower level of starvation resistance of introduced microbes, and lack of suitable soil niches for extended cell survival. Moisture content, pH, texture, and oxygen and nutrients availability are abiotic factors controlling the survival of microorganisms.

Encapsulation has emerged as a promising solution to overcome practical limitations of using free cell formulations. The polymeric matrix of the support material provides a defined, stable, consistent, and protective microenvironment, where cells can survive and metabolic activity can be maintained for extended periods of time without the immediate release of large number of cells. The entrapped cells can better tolerate numerous environmental stresses, and may be released after adaptation to surrounding environmental conditions. The main characteristics of microbial supports for environmental applications are summarized in **Table 1**. A variation of these characteristics may be considered in the selection of an appropriate microbial support for a specific application. For instance, micrometer-sized carriers are required for subsurface injection ***(33)*** whereas macrometer-sized supports may be desired for surface bioreactor applications or for constructing subsurface permeable reactive barriers ***(34)***.

The use of encapsulated cells for biodegradation of petroleum and gasoline hydrocarbons has been explored in a number of studies, as listed in **Table 2**. However, most of these investigations have been performed at the laboratory scale, and practical uses of encapsulated cells in the open environment have yet to be realized. Both natural and synthetic polymer gels have been used for microbial encapsulation. Criteria for gel supports to be used for soil applications are different from those required for controlled bioreactor systems. Although the long-term stability, low porosity, and small pore sizes afforded by synthetic polymers can be

Table 2
Examples of the Use of Encapsulated Microbial Cells in Biodegradation Studies

Compound	Microorganism	Carrier	Ref.
Petroleum			
Crude oil	Mixed culture	Urea-formaldehyde	***38***
Crude oil	*Yarrowia lipolytica*	Agar-Alginate	***39***
Crude oil	*Yarrowia lipolytica*	Polyurethane	***40***
Petroleum products			
Gasoline	Mixed culture	Gellan Gum	***41***
Aliphatic hydrocarbons			
Hexadecane	Mixed culture	Urea-formaldehyde	***38***
n-Alkanes (C_{14}-C_{16})	*Prototheca zopfi*	Alginate	***42***
Monoaromatic hydrocarbons (MAHs)			
Benzene	*Pseudomonas putida*	Polyacrylamide	***43***
Benzene	*Rhodococcus* spp.	Alginate	***44***
Polyaromatic hydrocarbons (PAHs)			
Naphthalene	*Pseudomonas* spp.	Alginate	***45***
Phenanthrene	*Pseudomonas* sp.	Alginate	***46***
Phenanthrene	*Pseudomonas* sp.	Alginate	***47***
Phenanthrene	Mixed culture	Urea-formaldehyde	***38***

effective for use in bioreactors, they may not be desirable in open environmental applications. The pore size of the support applied to a bioreactor process should be much smaller than the encapsulated cell to avoid or minimize cell release and washout from the bioreactor. Thus, cells remain inside the support while substrates and nutrients can diffuse in and products can diffuse out. In field applications, however, supports with high porosity are desirable because they provide high cell mass loading as well as high diffusional mass transfer rates. Therefore, the use of natural polymers with relatively larger pore size and greater degree of biodegradability may be suitable for some subsurface applications. It may be desired to release the encapsulated cells after positioning them in the target zone, and after adaptation to the surrounding environment ***(35)***. In general, natural polymers are recommended for use in soil. The characteristics of encapsulated inoculants and their advantages over free cells for use in soil have been discussed comprehensively in earlier reports ***(36,37)***.

Emulsion techniques have been utilized for the encapsulation of viable cells within micro- (0.1–1 mm) and macrospheres (1–3 mm) of natural thermotropic gel polymers such as agar ***(48)***, agarose ***(48,49)***, k-carrageenan ***(48,50)***, and gellan gum ***(51)***. The sphere formation process involves the dispersion of two immiscible liquid phases resulting in a water-in-oil (w/o) emulsion. A suspension of viable cells in an aqueous solution of the polymer (dispersed phase) is emulsified in a hydrophobic phase such as a vegetable oil (continuous phase). The gelation of the

small droplets of the dispersed phase is subsequently initiated by decreasing the emulsion temperature below the sol-gel transition temperature.

Other researchers have used emulsion techniques, termed as emulsification-internal gelation, to encapsulate biocatalysts ***(52,53)***, and DNA ***(54)*** in alginate microspheres. The gelation of alginate droplets was triggered by gentle acidification (to pH 6.5) of the water-oil dispersion through adding an oil-soluble acid and releasing soluble calcium ions from a salt complex.

This monograph outlines an emulsification-internal gelation method for encapsulation of gasoline-degrading bacteria in gellan gum microspheres. Calcium is used as the gelling agent and is available inside gellan gum sol droplets to induce an internal gelation.

2. Materials

2.1. Reagants

1. Microorganisms: A mixed bacterial culture in frozen or freeze dried state, capable of degrading gasoline hydrocarbons, for instance pre-isolated from a gasoline-contaminated soil and enriched in a mineral salts medium containing gasoline as a single source of carbon.
2. Mineral salts medium (MSM): an aqueous medium containing in g per liter: KH_2PO_4 0.87, K_2HPO_4 2.26, $(NH_4)_2SO_4$ 1.1, and $MgSO_4$ 0.047, and supplemented with 1 mL/liter trace metal solution composed in g/liter of: $Co(NO_3)_2 \cdot 6H_2O$ 0.291, $AlK(SO_4)_2 \cdot 12H_2O$ 0.474, $CuSO_4$ 0.16, $ZnSO_4 \cdot 7H_2O$ 0.288, $FeSO_4 \cdot 7H_2O$ 2.78, $MnSO_4 \cdot H_2O$ 1.69, $Na_2MoO_4 \cdot 2H_2O$ 0.482 and $Ca(NO_3)_2 \cdot 4H_2O$ 2.36. The pH of trace metal solution is lowered to about 2.0 by adding a small amount of a 2 *N* HCl for complete dissolution of minerals. Mineral salts medium is sterilized at 121°C for 20 min. The final pH of the medium is 7.0 ± 0.1.
3. Gasoline: an unleaded product with an octane number of 87 purchased from a commercial fuel distributor.
4. Gellan Gum: Gellan gum (Kelcogel®, CP Kelco US, Inc., San Diego, CA) is an extracellular polysaccharide produced through aerobic fermentation processes by the microorganism *Sphingomonas paucimobilis* ATCC 31461 ***(55)***, earlier referred to as *Pseudomonas elodea* ***(56,57)***, *Auromonas elodea* ***(58)***, and *Sphingomonas elodea* ***(59)***. The chemical structure of gellan gum (*see* **Fig. 1**) is made up of repeating tetrasaccharide units consisting of a linear sequence of D-glucose, D-glucuronic acid, D-glucose, and L-rhamnose ***(60,61)***.

   The mechanism of gelation and texture of gellan gum suggests a strong similarity with agar and carrageenans. However, gellan gum gel has superior rheological properties to agar and carrageenan gels at equivalent concentrations ***(62)***, and therefore, it can be used at substantially lower concentrations. The gel has been stable over the wide pH range of 2.0 to 10.0 ***(63)***, suggesting its suitability for use in both acidic and basic environments. The application of gellan gum for encapsulation of viable cells has been addressed at considerably lower concentrations of both gel and gelling agent compared to κ-carrageenan, agar, and alginate ***(48,64)***. Unlike some other ion-sensitive gelling polysaccharides such as alginate and κ-carrageenan, the reactivity between gellan gum and ions is non-

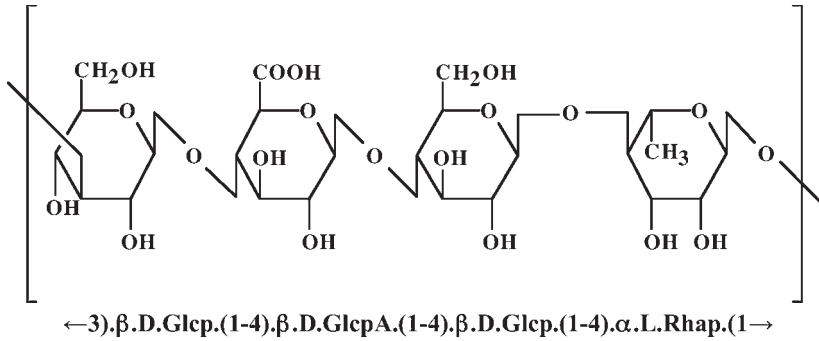

Fig. 1. Gellan gum repeating unit ***(60,61)***.

specific and gels can be formed with a wide variety of cations including alkaline and alkaline-earth cations ***(65,66)***.

5. Canola oil A food-grade product purchased from a local food distributor, sterilized at 121°C for 20 min.
6. Sorbitan monoleate, Span®80.
7. Polyoxyethylene [20] sorbitan monooleate, Tween®-80, A 0.1% (v/v) solution is prepared in distilled water sterilized at 121°C for 20 min.
8. Calcium chloride: A 0.1% (w/v) solution is prepared in distilled water sterilized at 121°C for 20 min.
9. Sodium hydroxide: A 0.1 *N* solution is prepared in distilled water.
10. Hydrogen chloride: A 2 *N* solution is prepared in distilled water.
11. De-ionized water sterilized at 121°C for 20 min.
12. Ice/water bath.

2.2. Equipment

1. Round-bottomed cylindrical reaction vessel, 10-cm diameter × 1 L capacity, equipped with a standard four-blade baffle
2. Quarter-circular paddle impeller, 5-cm diameter.
3. High shear mixer, 5000 rpm (e.g. ,T-line Laboratory Stirrer, model 102, Talboys Engineering Corp., Montrose, PA).
4. Photo/contact digital tachometer.
5. Hot plate/magnet stirrer.
6. Magnet stirrer.
7. Graduated plastic 50-mL centrifuge tubes with screw caps.
8. Centrifuge and rotor suitable for 50-mL centrifuge tubes.
9. Shaker/incubator.
10. Tap water vacuum ejector with connector tube and plastic conical tip.
11. 1000 mL Erlenmeyer flasks.
12. 100 and 1000 mL Graduated cylinders.
13. 80, 500, and 1000 mL Beakers.
14. All glassware and equipment in contact with bacteria are sterilized at 121°C for 20 min.

3. Methods

See **Note 1**.

3.1. Preparation of Enrichment Culture

1. A given amount of mixed bacterial culture is suspended in 250 mL mineral salts medium supplied with gasoline at 100 to 150 mg/L, as the sole source of carbon, in a 1000-mL Erlenmeyer flask. The initial number of cells may range from10^8 to 10^9 colony forming units (CFU)/L of medium.
2. The enrichment can be done through multiple successive transfers before harvesting the cells for encapsulation.

3.2. Cell Harvesting

1. The cells are harvested by transferring the enriched culture into centrifuge tubes and centrifugation at 12,000*g* for 15 min at 4°C. Cell pellets are collected and resuspended in 2 mL calcium chloride solution.

3.3. Preparation of Gellan Gum Pregel Solution (Dispersed Phase)

1. Gellan gum is dispersed in 100 mL de-ionized water at a concentration of 0.75% (w/v).
2. The dispersion is heated to between 75°C and 80°C while being stirred on a hot plate/magnet stirrer to dissolve gellan gum.
3. Calcium chloride is added to the solution at a concentration of 0.06% (w/v).
4. The resulting pregel solution is then left at room temperature to cool to 45°C.

3.4. Incorporation of Bacteria

1. The pH of pregel solution is adjusted between 6.9 and 7.2 with 0.1 *N* NaOH before incorporation of bacteria.
2. The suspension of bacteria in calcium chloride solution (*see* **Subheading 3.2.**) is then added to the pregel solution.
3. The mixture is agitated for 2 to 3 min for complete homogenization.
4. The final concentration of bacteria may range from 10^9 to 10^{10} CFU/mL (*see* **Note 2**).

3.5. Preparation of Canola Oil (Continuous Phase)

1. Span® 80 is admixed with 300 mL canola oil at 0.1% (w/w) in the reaction vessel (*see* **Note 3**).
2. The oil is heated to 45°C on a hot plate while being stirred using the high shear mixer.
3. The impeller is adjusted at one-sixth of the liquid height from the reactor bottom.
4. The impeller speed is set at 4500 rpm by means of a tachometer and re-adjusted during operation if necessary.

3.6. Emulsification

1. Homogenized bacteria suspension (*see* **Subheading 3.4.**) is emulsified in canola oil (*see* **Subheading 3.5.**). The resulting w/o emulsion is stirred for 3 min to achieve homogeneity (*see* **Notes 4–6**).

3.7. Gelation

1. The gelation of gellan gum is triggered by cooling the reaction vessel to about 15°C using the ice bath, leading to formation of gellan gum-encapsulated cell microspheres.

3.8. Separation of Microspheres

1. The oil-microsphere dispersion (*see* **Subheading 3.7.**) is transferred with gentle mixing into 500 mL of calcium chloride solution.
2. The oil is removed by aspiration using the tap water ejector after partitioning of microspheres into the aqueous phase.
3. The microspheres are rinsed repeatedly with the Tween®-80 solution , and stored in calcium chloride solution at 4°C.

4. Notes

1. Even though the materials and methods described in this monograph are used for the encapsulation of gasoline-degrading bacteria, they may be applicable to encapsulation of other microbial degraders in macro- or microspheres subject to the employment of proper emulsification conditions.
2. Concentration of emulsifier, Span® 80, in the continuous phase may vary from 0 to 0.15% (w/w) to obtain different final particle size distributions.
3. Emulsification-internal gelation method described in this monograph allows encapsulating bacteria at a cell loading range of, but not limited to, 10^9 to 10^{10} CFU/mL microsphere.
4. Emulsion stirring speed may vary from 1000 to 5000 rpm to obtain different final particle size distributions.
5. Dispersed phase volume ratio (the ratio of dispersed phase volume to total emulsion volume) may be adjusted between 0.077 and 0.25 to obtain different final particle size distributions.
6. Emulsification time may be set between 1 and 10 min to obtain different final particle size distributions.

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Biomedical Applications of Immobilized Cells

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Summary

The aim of cell microencapsulation technology is to treat multiple diseases in the absence of immunosuppression. For this purpose, cells have been immobilized experimentally within carefully designed capsules that allow the long-term function of the graft. Recently, several advances have brought the whole technology much closer to a realistic proposal for clinical application. This chapter reviews the potential impact of this alternative approach for the long-term delivery of therapeutic products and discusses the main limitations, advantages, and challenges in order to assure the same quality standards as those associated with approved drug delivery systems.

Key Words: alginate; cell encapsulation; drug delivery; immobilization; microcapsules.

1. Introduction

Many approaches have been developed over the past decades that have set the stage for tissue and organ replacement as well as for the continuous and controlled release of therapeutic agents to the host. Encapsulation of living cells is one of these technologies in which cells are enclosed within semipermeable encapsulation systems fabricated both by natural or artificial polymers designed to circumvent immune rejection (*see* **Fig. 1**) ***(1)***. In general, this biotechnology strategy has two important therapeutic potentials: on the one hand the long-term transplantation of biologically active molecules to restore or improve native tissue function and on the other hand the development and optimization of novel drug delivery systems that would allow the long-term secretion of the therapeutic products. Both approaches have significant impact from a therapeutic and economic standpoint. In fact, as the technologically optimized encapsulation system would prevent antibodies and/or other immune cells from entering and destroying the encapsulated cells, chronic administration of immunosuppressant drugs might be reduced, thereby facilitating a better quality of life for patients undergoing this treatment.

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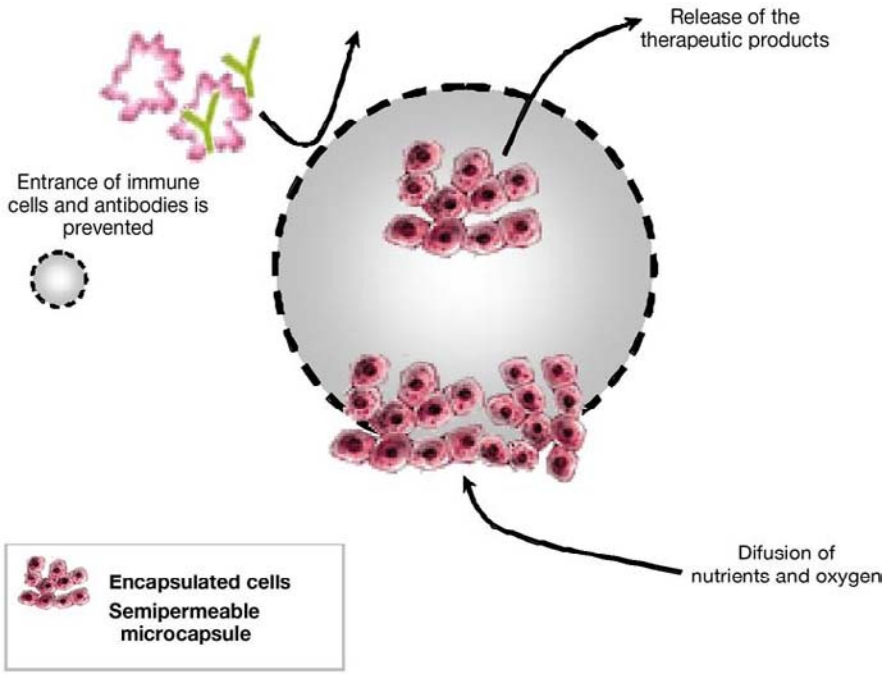

Fig. 1. The semipermeable membrane of the microcapsules allows the bidirectional diffusion of nutrients, oxygen and waste, yet prevents immune cells and antibodies, which might destroy the enclosed cells, from entering.

The initial attempts in transplanting cells embedded in polymers dates back to 1933, when Bisceglie decided to introduce tumor cells in a polymer structure and transplant it into a pig's abdominal cavity ***(2)***. In the 1960s, the concept of "artificial cell" was invoked to refer the immuno-isolation of cells and enzymes within semipermeable microcapsules ***(3)***. Since then, tremendous efforts have been made all around the world to advance the understanding of biology, genetics, polymer science, and pharmaceutical technology. More sophisticated and technologically optimized microcapsules have been fabricated to improve the viability and functionality of the enclosed cells as well as to resist the handling, transplantation, and stress inside the host. In addition, many different cell sources have been immobilized and assayed as potential drug-secreting factories. From all these studies, we now know that clearly not all cells are suitable for microencapsulation. Cells that proliferate following encapsulation could eventually fill the entire capsular space and lead to diminished efficacy of therapeutic diffusion, which could compromise the long-term viability of immobilized cells. In contrast, cells that do not proliferate after encapsulation, such as myoblasts, have the potential to deliver therapeutic products for longer periods of time.

Interestingly, since the pioneering approaches put in practice in the 1980s to immobilize xenograft islet cells in alginate-poly-L-lysine (PLL) microcapsules to

aid in glucose control for diabetes in small animal models ***(4)***, the use of this biotechnological strategy has extended to novel therapeutic objectives. In fact, its proof of principle as a drug delivery sytem and allogeneic transplantation has been successfully demonstrated in animal models for a wide range of diseases, including cancer ***(5)***, hemophilia ***(6)***, and renal failure ***(7)***.

2. Microencapsulation Process: Polymers and Cells

In cell microencapsulation technology the correct combination of the two main components, cells and polymers, is totally necessary if the success of this strategy is pretended. Many natural and synthetic polymers have been assayed both for capsule matrix and outer membrane. All these materials must be biocompatible, which means that they must not interfere with enclosed cell homeostasis nor induce a host immune response that will reduce the long-term functionality of the transplanted microcapsules ***(8)***. Furthermore, the microcapsules fabricated with these polymers must present enough stability to resist mechanical stress and osmotic stress once implanted. Therefore, it is essential to select correctly the materials that will take part in the final structure of the immobilization device.

Alginate (composed by mannuronic and guluronic dimers) is the most frequently employed material reported in the scientific literature, in part because of its excellent biocompatible and biodegradable properties. A large number of experiments have focused on identifying the effects of alginate composition, purity, and concentration on the viability and peptide production of the enclosed cells ***(9,10)***. In general, it is assummed that purification of the alginates is a priority for ensuring their biocompatibility, while the suitability of monomer composition of the capsules is still a matter of debate. Recently, novel materials have come under development, including oligochitosans, cellulose sulfate, pectins, and different synthetic polymers. Coating of the fabricated capsule matrix with a suitable semipermeable membrane is another emerging area of research. This outer membrane will allow the bidirectional diffusion of nutrients, oxygen, and waste, while the entrance of immune cells, antibodies, and other components of the immune response will be prevented. Alginate-PLL complex is the most studied membrane chemistry but other polycations such as poly(ethylene glycol) (PEG), polyvinylalcohol (PVA), and poly-L-ornithine (PLO) have also been evaluated ***(11)***.

A suitable choice of encapsulated cells is key for the success of any biomedical application. A large number of cells have been studied as potential candidates for long-term secretion of therapeutic products as shown in **Table 1**.

After the polymers and therapeutic cells have been selected, technologically optimized microcapsules must be fabricated in order to obtain a functional drug delivery system. The choice of the microencapsulation technology as well as the microencapsulation system will rely on the selected biomaterials. Therefore, totally different fabrication procedures will be used if natural or synthetic polymers are chosen. In the case of alginate–PLL–alginate microcapsules, the most widely used immobilization devices for cell entrapment, polymeric devices are produced at room temperature and sterile conditions by an ionic gelation method using an extrusion system. Initially, therapeutic products secreting cells are suspended with the sodium alginate (usually 1.5% [w/v] or 2% [w/v]). This cell–gel suspension is extruded into a gellifying solution such as calcium chloride or barium chlo-

Table 1
Examples of Cells Selected for Immobilization Purposes

Immobilized cells	
Parathyroid cells	Fibroblasts
Hepatocytes	Myoblasts
Chondrocytes	Kidney cells
Leydig cells	Hybridoma
Adrenal chromaffin cells	Tumour cells
Stem cells	Virus producer cells
PC12 pheochromocytoma	Bacteria
Enzymes	Neurons

ride. The contact of the sodium alginate with the gellifying solution provokes the polymerization of the matrix because of the ionic change leading to calcium or barium alginate microbeads ***(12)***.

Upon total gelation, cell loaded microbeads are chemically cross-linked with PLL and after a washing step, the beads are coated with another layer of alginate (*see* **Fig. 2A**). The latter is necessary to address the problem of immune rejection because the polycations used to form the semipermeable membrane attracts inflammatory cells inducing necrosis of the encapsulated cells ***(13)***. Once fabricated, the microcapsules can be used in the treatment of several diseases (*see* **Fig. 2B**).

3. Microcapsule Evaluation

One priority in the field of cell microencapsulation is the validation and optimization of the biomaterials and technology used in the fabrication of the immobilization systems as well as the assays and protocols needed to analyze and reproduce the devices. The large number of techniques and assays that have recently been refined have opened new avenues in the systematization of this therapeutic strategy. The purity and biocompatibility of the materials is a matter of much research. For example, several assays have been validated for purification of alginates, including the use of chemical regents and dialysis ***(14)***, the induction of apoptosis in Jurkat cells, and the lymphocyte proliferation assay. The latter is a rapid and sensitive screening for any fibrosis-inducing impurities in alginate samples ***(15)***. Production of high levels of cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and IL-6 from human monocytes stimulated by alginates of different composition has also been reported ***(16)***.

The preliminary study of the biomaterials must be followed by a morphological evaluation and in vivo biocompatibility of the microcapsules. To address these issues of techniques across a wide spectrum have been optimized, such as confocal laser scanning microscopy (CLSM), advanced nuclear magnetic resonance (NMR), atomic force microscopy (AFM), Fourier transform infrared spectroscopy (FT-IR), and the X-ray photoelectron spectroscopy analysis. All these approaches allow for a detailed analysis of the microcapsules and their components. The CLSM method is employed to study the distribution of the alginate and PLL in

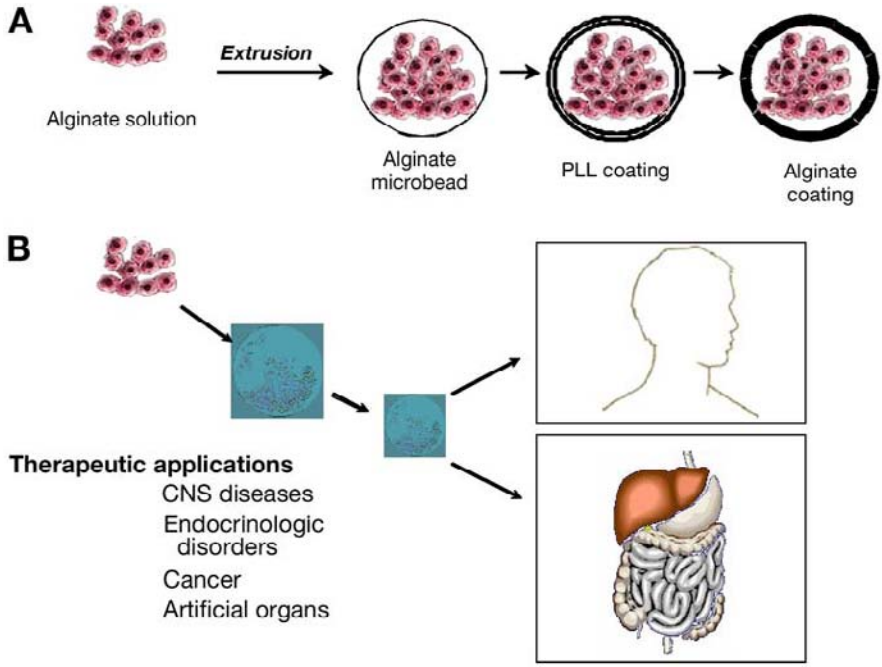

Fig. 2. **(A)** Elaboration of alginate-PLL-alginate microcapsules for cell immobilization. **(B)** Therapeutic applications of microencapsulated pharmacologically active cells.

intact microcapsules ***(17)***, while the combination of NMR and AFM is used to give clear-cut evidence of the capsular surface topography and the cross-linkage characteristics between alginate and the gellifying ion ***(18)***. Finally, FT-IR and the X-ray photoelectron spectroscopy are used to evaluate the reaction against the microcapsules in the immediate posttransplant period and to determine the pivotal factors for producing biocompatible capsules ***(19,20)***. In addition, the detailed histological and immunopathological analysis of the implanted microcapsules ***(21)*** as well as their in vivo antigenicity evaluation ***(10)*** are exciting tools to study the biocompatibility of the immobilization devices.

In the quest for stronger microcapsules, several tests that measure the mechanical stability of the particles have been designed. The resistance of the microcapsules against compression can be measured using a texture analyzer system ***(22)***, while a more complete idea of the mechanical properties of the polymer systems might be obtained by exposing the particles to destabilizing forces including swelling solutions and shear forces ***(23)***. A novel osmotic pressure test has been developed to quantify the strength of the microcapsules by exposing them to a graded series of hypotonic solutions and quantifying the percentage of the broken capsules ***(24)***. Additionally, high-frequency scanning acoustic microscopy (SAM) has been successfully employed to characterize the mechano-elastic parameters and surface structure and topography of alginate microcapsules ***(25)***.

Another focus of interest is the evaluation of the permeability properties (ingress and egress behavior) of the semipermeable microcapsules. The feasibility of a membrane depends on how appropriate is the control over both the size-based exclusion and the rate of diffusion of the molecules, which either have to or must not permeate the membrane to control the survival as well as the metabolic efficacy of the graft. Generally, the process of transport of species across a membrane, characterized as the membrane permeability, is governed by both the thermodynamic parameter known as equilibrium partition coefficient and the kinetic parameter known as diffusion coefficient. A number of assays have been refined to characterize the permeability properties, including the permeability to hemoglobin and immunoglobulin G (IgG) ***(26)***, of the immobilization devices: size exclusion chromatography using dextran standards of different molecular weights ***(27)*** and the determination of the mass transfer coefficient value ***(28)***. The latter has been succesfully employed in the preliminary screening of microcapsules composed of three different membrane chemistries: PLL, poly-L-ornithine (PLO), and poly-methylene-co-guanidine hydrochloride (PMCG) ***(29)***.

In the future, It seems to be advisable that the optimization of encapsulated cells will involve assessing the mechanical stability, diffusion, and permeability properties in vivo. These challenges will be crucial for the standardization of immuno-isolation devices for the pre-Food and Drug Administration stage.

4. Therapeutic Applications

The wide range of therapeutic applications of cell microencapsulation technology can be classified in five groups: (1) treatment of classical Mendelian disorders, (2) cancer treatment, (3) central nervous system (CNS) diseases, (4) artificial organs and, (5) others. Some of the most interesting approaches are described in **Table 2**.

In light of the current desperate shortage of donor organs, both allogeneic and xenogeneic cells and tissues have been tested as potential living drugs. Xenografts, however, have become a major cause of concern because of the possible transmission of infectious agents, particularly the porcine endogeneous retrovirus (PERV), from the donor to recipient ***(45)***. Additionally, the construction of genetically modified cells has opened new avenues for the treatment of classical Mendelian disorders and cancer. In essence, genes can now be used as templates, cells as reactors to secrete the final product and capsules as immunoisolation vehicles for drug delivery in vivo. Another important consideration in using genetically manipulated cells is biosafety. In fact, safety and stability of gene expression will have to be balanced in the use of genetically modified cells.

The results achieved from small and large animal models have provided the scientific basis for several clinical trials, including the encapsulation of allogeneic islets for the treatment of diabetes ***(46)***, the encapsulation of cytochrome P450 enzyme expressing cells for the eradication of pancreatic cancer ***(47)***, or the immobilization of retinal pigmented epithelial (hRPE) cells on gelatin microcarriers to treat patients with advanced Parkinson's disease.

5. Recent Advances in Cell Encapsulation Technology

Because the fields of gene therapy, cell biology, pharmaceutical technology, and chemical engineering are developing rapidly, scientists are continually report

Table 2
Therapeutic Applications of Cell Microencapsulation Technology

Disorder/application	Cell line	Therapeutic product	Ref.
Spinar cord injury	Fibroblasts	BDNF	***30***
Hemophilia B	C_2C_{12} myoblasts	Factor IX	***31***
Hemophilia A	C_2C_{12} myoblasts	Factor VIII	***32***
Cancer	Fetal kidney cell	Nitric oxide	***33***
MPS VII	2A-50 fibroblasts	β-glucuronidase	***34***
Anemia	Human fibroblasts	EPO	***35***
Parkinson's disease	C_2C_{12} myoblasts	GDNF	***36***
Cancer/antiangiogenesis	C_2C_{12} myoblasts	Angiostatin	***37***
Cartilage repair	C3H10T1/2 cells	BMP-2/BMP-4	***38***
Diabetes	Islets of Langerhams	Insulin	***39***
Parkinson's disease	PC12 cells	Dopamine	***40***
Therapeutic angiogenesis	Primary mioblasts	VEGF	***41***
Hypoparathyroidism	Parathyroid tissue	Parathyroid hormone	***42***
Cancer/antiangiogenesis	BHK-21 cells	Endostatin	***43***
Cancer	Hibridoma	Anti-VE cadherin antibodies	***44***

BDNF, brain-derived neurotrofic factor; BHK cells, baby hamster kidney cells; BMP, bone morphogenetic protein; EPO, erythropoietin; GDNF, glial-cell derived neurotrofic factor; MPS, mucopolysaccharidosis; VE, vascular endothelium; VEGF, vascular endothelial growth factor.

new applications based on cell microencapsulation technology. For example, immobilized cells secreting granulocyte macrophage-colony-stimulating factor (GM–CSF) are being studied for immunomodulating or adjuvant activity in the context of immunization in humans and animals. These live vaccines might be suited for both cancer immunotherapy and vaccination against infectious diseases.

Another application consists on the immobilization of engineered cells secreting therapeutic antibodies. Several cell types have been used for this approach, including hybridomas, skin fibroblasts, keratinocytes, myogenic cells, and hepatocytes. Interestingly, in some cases the antibodies produced both in vitro and in vivo, retain the specificity and the affinity of the parenteral antibody and no anti-idiotypic response is detected in animals producing ectopic antibodies ***(48)***. Similarly, as repeated delivery of a vector is necessary to ensure adequate transfer of the therapeutic gene, encapsulated cells producing retroviral vectors have been evaluated for long-term in vivo gene transfer ***(49)***.

Scientists are making great efforts in the development of a bioartificial pancreas by immobilizing pancreatic islets in polymer microcapsules. Some research groups have concentrated on creating a neovascularized site where the encapsulated islets can be implanted. Although not fully supported by all experts, this approach will, in theory, facilitate the contact between the blood-stream and the immobilized cells and this could be beneficial for their long-term performance and

functionality ***(50,51)***. Such a strategy can be easily performed by coencapsulating angiogenic factor-secreting cells or using basic fibroblast growth factor (bFGF) or vascular endothelial growth factor-impregnated gelatin microspheres ***(52)***.

6. Conclusions and Perspectives

Cell microencapsulation is gaining attention as an alternative strategy to conventional therapeutic treatments. However, numerous challenges remain in this technology including developing biomaterials with higher biocompatibility and purity, fabricating microencapsulated systems with higher reproducibility and biosafety, and testing novel cell lines with improved functionality once immobilized. The ability to address all these challenges will potentiate this technology and extend its therapeutic applications.

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